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Calling Attention To 700

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PHENYLSERINE STUDIES.
II. CHANGES IN BACTERIAL SENSITIVITY TO DL–THREO–PHENYLSERINE*

ERNEST BEERSTECHER, JR.

The first paper in this series (Beerstecher, Edmonds, and Volkmann, 1955) has summarized the reasons for broad general interest in the mechanism of action of phenylserine and its derivatives on biological systems. Beerstecher and Shive (1947) concluded that phenylserine blocked the conversion by *Escherichia coli* of phenylalanine to some unknown product necessary for growth, but not to tyrosine. Molho-Lacroix and Molho (1952a), however, reported that phenylalanine specifically inhibited the conversion of phenylalanine to tyrosine, and that once the organism becomes adapted to phenylserine, it can make tyrosine without the use of phenylalanine as an intermediate. They also found (1952b) that chloromycetin-resistant strains of *Escherichia coli* tend to become more sensitive to phenylserine. In an effort to resolve these and other similarly conflicting bits of evidence bearing on the subject, studies have been made on the effects of culturing the test organism in a phenylalanine-containing medium.

Experimental

The strain of *Escherichia coli* used, the medium and the test methods were the same as previously described (Beerstecher and Shive, 1946). The organism was transferred from a nutrient agar slant into a mineral salts-glucose medium containing 1.0 mg. per ml. of DL-phenylalanine. At twenty-four hour intervals a loop of the culture was transferred to a fresh tube of this medium, and the serial transfers were continued in this manner for a two-week period, after which time the culture became rough and no longer suitable for testing. At two- or three-day intervals the culture was tested for its sensitivity to DL-*threo*-phenylserine in the absence of added DL-phenylalanine, and in the presence of DL-phenylalanine at levels of 1.0 ug. per ml. The results are summarized in table 1, which shows that serial transfer in a phenylalanine-containing medium increases the sensitivity of *Escherichia coli* to phenylserine, but that the ratio of phenlyserine to added phenylalanine which is required to give fifty per cent inhibition of growth remains constant. The

* From the Department of Biochemistry, The School of Dentistry, The University of Texas, Houston. Received for publication May 3, 1955.

same result is obtained when the culture is transferred through medium containing as low as 10 ug. per ml. of phenylalanine, but not through phenylalanineless medium. The inhibitor-substrate ratio also remains constant with successive transfers when the organism is *tested* in the presence of other levels of added phenylalanine ranging from 0.2 to 5.0 ug. per ml. medium. Within the latter range of concentrations, the ratio is independent of phenylalanine concentration, as would be expected if the two substances were competing for some single enzyme system which converts phenylalanine to some other product essential for growth (Beerstecher and Shive, 1946).

Discussion. The increased phenylserine sensitivity of *Escherichia coli* when cultured in the presence of phenylalanine might be explained either on the basis of decreased phenylalanine-synthesis by the organism, or decreased synthesis of phenylalanine-utilizing enzyme. If the latter is true, then it should follow that

TABLE 1

Relationship of Serial Transfers in Phenylalanine-Containing Medium to Phenylserine Senstivity of Echerichia coli

No. of Daily Serial Transfers in Phenylalanine Medium	Log. concentration (mg./10 ml.) to produce 50 Percent Inhibition of Growth	
	Tested in Plain Medium	Tested in Plain Medium + 1 ug./ml. DL-phenylalanine
3	...	3.48
4	3.25	...
8	2.78	3.53
9	2.55	3.50
10	2.33	...
12	2.03	3.50
13	1.90	...
15	...	3.55

the antimetabolite would manifest increased effectiveness against phenylalanine. Since, however, the ratio of inhibitor to substrate remains constant, it seems most probable that the enzyme concentration within the bacterial cells is not changed under these conditions. This conclusion agrees with the observations of other workers with other types of antimetabolites. Decreased synthesis of phenylalanine thus seems to be the best explanation of the observed phenomena. Separate plating studies show that many cells actually lose their phenylalanine synthesizing abilities, but that the phenylalanineless "mutants" so formed revert to the wild type when serially transferred in suboptimal concentrations of phenylalanine.

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THE ANESTHETIC MANAGEMENT OF THE AGED (A REPORT OF 5450 CASES)*

GUNTER CORSSEN

During the last fifteen years, interest in the problems of surgical and anesthetic management of aged people has shown a remarkable increase. The ever-growing number of aged people following a steady increase of the life span in all civilized countries of the world during the last half century is naturally associated with a corresponding increase in the number of aged patients needing medical care.

More adventurous surgery as well as the rapid development of new anesthetic methods, improvement of already existing techniques together with the use of more powerful and safer drugs and the combination of different techniques in "balanced anesthesia" made it possible to develop a particular field of anesthesia for the benefit of the aged.

The anesthetic management of elderly people confronts us with many specific problems. Accordingly, numerous authors have reported on this subject earlier in attempting to cover thoroughly the problems involved (Lawrence, 1939; Burdick, 1942; Baird, 1943; Hershey and Apogi, 1944; Lawrence, 1945; Rink, Turville and Dripps, 1948; Lorhan, Goffen and Wood, 1949; Foldes, Gilette, 1950; Johnson and Livingston, Wilson, 1952; Bernstine and Golden, Sadove and Kahan, Ziffren and Sheets, 1953; Lautner, 1954). The over-all summary of these publications reveals the significant roles which particular preoperative and preanesthetic care, proper selection of anesthetic technique, skillful and cautious administration of the chosen drug and special postoperative care play in geriatric anesthesia.

Several observers have focused particular attention on the patho-physiological changes in the aged (Knight, 1944; Watts, 1945; Rovenstine, 1946). They all agree that people of advanced age usually show a lower metabolism and a slower absorption and delayed elimination of any given drug. These factors are consid-

* From the Department of Anesthesiology, The University of Texas Medical Branch, Galveston. Received for publication May 5, 1955.

ered to be especially important in connection with the use of barbiturates, opiates, or demerol, for premedication prior to surgery or for sedation in combating pain during the postoperative course.

It has been stated that age alone is not a sound criterion on which to base anesthetic management. Therefore, aged people should not present necessarily greater surgical risks, even if we consider that old age is usually accompanied by a decrease in the adaptive power of the body system to any stress with a lowered elasticity of the vascular system, a generalized atrophy of the muscles, including those of the heart, and a certain weakness of the central autonomic control.

In all previous publications related to geriatric anesthesia relatively little specific information was given regarding preoperative morbidity and physical state of aged patients facing anesthetics for surgical procedures. Intra- or postoperative complications possibly connected with or favored by the anesthetic agent or method had not been adequately investigated. Little knowledge seemed to exist also concerning the influence of the length of operation and anesthetic on the occurrence of complications during the procedure or in the postanesthetic period. Therefore, we felt it worthwhile to study these problems more thoroughly and to attempt to demonstrate the particular circumstances under which we conduct anesthetics for the aged. This may be beneficial to anticipate and perhaps avoid severe and fatal complications.

In this 12 year study (1942–1953), we review anesthesia records of patients aged 60 years and older undergoing surgery and receiving a general or spinal anesthetic. The total number of all anesthetic procedures during this period was 46,253. Five thousand four hundred fifty anesthetics were given to 5,075 patients 60 years of age and older. This was 11.8 percent of the total number of patients. Figure I indicates the rapid and progressive increase of the number of geriatric anesthetics during the period covered by this survey. Our files reflect only 165 anesthetics to individuals of this type in 1942. In 1953, in comparison, we performed 743 anesthetics on elderly patients which discloses that the number of geriatric anesthetics increased 4½

times by the end of this period. At the same time, the number of all administered anethetics was only doubled.

The total number of patients was 5,075. Of this number there were 3,622 males (71.4 percent and 1453 females (28.6 percent). The greater number of operations on male patients is explained by the role which the urology service plays in aged males.

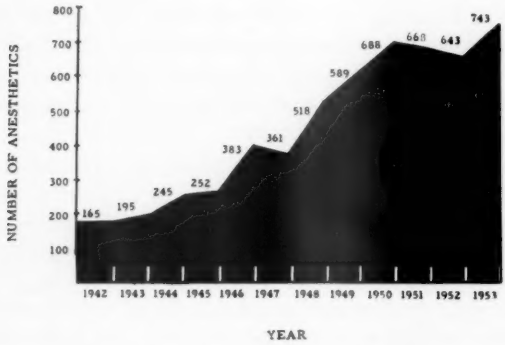

FIG. I. Geriatric anesthetics, 1942–1953.

The age distribution is demonstrated in table 1.

Three thousand four hundred forty-three patients (67.8 percent of the total number of patients) belonged to the group between 60 and 70 years of age; 1,632 patients (32.2 percent of the total number of patients) were over 70 years of age. Five patients, older than 90 years of age, ranged from 91 to 103.

A classification of our patients according to physical state as recommended by Saklad (1942) is given in figure II.

One thousand three hundred sixty-one patients (26.8 percent of the total number of patients) belonged to Physical States 1 and

TABLE 1

Age Distribution of 5,075 Patients

	60–65	66–70	71–75	76–80	81–85	86–90	91 and Over	Total
Total No. of Patients	1,978	1,465	890	508	173	56	5	5,075
Percent of Total No. of Patients	38.9	28.9	17.6	10.0	3.4	1.1	0.1	100

5 and seemed to be free of any morbid change. These individuals showed only the physiological, functional and structural changes of old age. Three thousand four hundred eighty-eight patients (68.7 percent of the total number of patients) belonged to Physical States 2, 3 and 4. According to the grading scale, these patients suffered from a moderate, severe or extreme systemic dis-

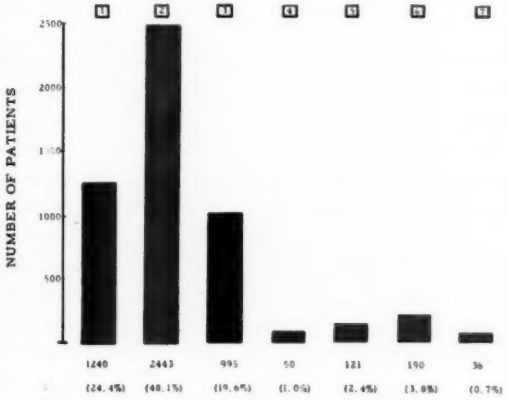

FIG. II. Physical state of 5,075 geriatric patients.

order. Three hundred forty-seven patients (6.8 percent of the total number of patients) underwent emergency procedures. Thirty-six of these patients belonged to Physical State 7 and came practically moribund to the operating room.

The preoperative morbidity on which the grading as to physical states was based is shown in table 2.

TABLE 2

Predominant Preoperative Complications in 5,075 Patients

	Hypertension (Syst. B.P. More Than 175 mm.)	Organic Heart Diseases	Pulmonary Complications	Toxemia	Generalized Marked Arteriosclerosis	Severe Anemia (Hb below 10 gm.)	Miscel.	Free of Any Systemic Disorder	Total
No. of Patients..	936	599	485	470	419	292	513	1,361	5,075
Percent of Total No. of Patients	18.4	11.8	9.5	9.3	8.3	5.8	10.1	26.8	100

For the purpose of this table only the leading symptoms or the most serious complication which characterized the patient's physical state was considered. Quite a few patients revealed, of course, more than one of the listed complications, which naturally increased the anesthetic risk.

Hypertension as a definite preoperative disturbance was only determined as such in combination with other systemic disorders, such as marked arteriosclerosis, degenerative heart disease, etc.

Patients showing either signs of decompensation or who were being treated with digitalis because of a previous period of decompensation were included in the group with organic heart diseases. To this group also belong those patients who revealed signs of a definite myocardial damage in the electrocardiogram.

In the group of pulmonary complications, patients with marked emphysema, bronchiectasis, active specific processes, atelectatic changes and pulmonary congestion were considered.

Among the patients with toxemic symptoms, we included those with moderate or severe renal impairment (increase of NPN over 50 mgm. percent, positive protein test in the urine, etc.) and hepatic or pancreatic disorder (pronounced jaundice, moderate or severe diabetes).

The distribution of the 5,450 operations to the different services is illustrated in figure III.

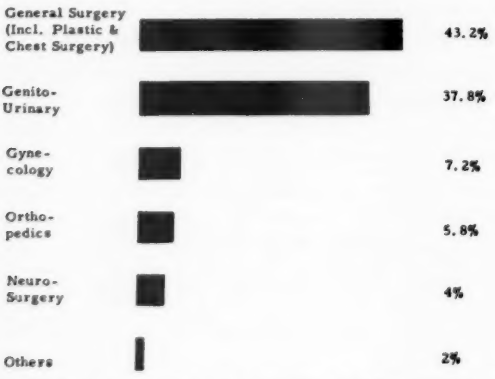

FIG. III. Distribution of 5,450 operations to different services.

In regard to choice of anesthesia method and selection of anesthetic agent, figure IV demonstrates the outstanding role which cyclopropane plays in our series.

A more detailed investigation of agents and methods is revealed in table 3.

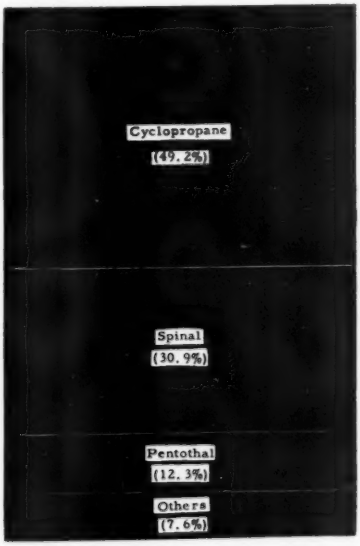

FIG. IV. Anesthetic agents and methods used in 5,450 operations.

TABLE 3

Anesthetic Agents and Methods Used in 5,450 Operations

	Cyclopropane (Alone or in combin. with N_2O or Ether (or both)	Spinal (Alone or with Gas)	Pentothal (Alone or with Gas)	Nitrous-Oxide-Oxygen	Ether (O.D.)	Local and Regional (Alone or with Gas)	Saddle Block, Trans-Sacral-Epidural Block	Avertin (Rect.) (With and Without Supp. Gas)	Oxygen	Refrig. (Alone or with Gas)	Curare and Curare like Drugs	Total
No. of Operations	2,698	1,683	671	140	101	91	38	15	10	3	289*	5,450

*Not included in Total.

A summary of the complications which occurred during the anethesia course is given in table 4. We considered here only disturbances which interfered with the anesthesia procedure by causing a significant deviation from the normal pattern.

The relation of the intraoperative complications to the anesthetic method and agent is summarized in table 5.

TABLE 4

Summary of Complications During the Anesthesia Course

BP Drop *Without* Signs of Circl. Distress	BP Drop *With* Signs of Circl. Distress	BP Rise (CO_2 Excess Hypoxia)	Severe Cardiac Arrhythmia Heart Block	Circl. Failure	Marked Respir. Impairment of Different Origin	Total
222	81	21	39	9	65	437

TABLE 5

Summary of Complications During the Anesthesia Course in Relation to Anesthetic Method or Agent

	Cyclo-propane	Spinal	Pento-thal	N_2O	Ether	Local and Regional Block, Local Refrig-eration	Avertin (Rect.)	Oxygen	Curare and Curare-like Drugs	Total
BP Drop Below 70 mm. syst. With and Without Signs of Distress	176	82	10	.	16	6	4	.	9	303
BP Rise Above 250 mm. syst. (CO_2 Excess, Hypoxia)	17	1	3	.	.	.	.	.	.	21
Severe Arrhythmia, Heart Block ...	24	5	5	1	2	2	.	.	.	39
Cardiac Arrest ...	6	.	.	1	1	.	.	1	.	9
Respiratory Impairment of Various Origins	45	3	14	.	2	.	1	.	.	65
Total No. of Complications .	268	91	32	2	21	8	5	1	9	437
Total No. of Administered Anesthetics	2,698	1,721	671	140	101	94	15	10	289*	5,490
Percent of Total No. of Anesthetics Related to Drug and Method ...	10%	5.2%	4.8%	1.4%	20%	8.5%	33.3%	10%	3.1%	8%

* Not included in Total Number.

A brief summary of the main complications which occurred postoperatively is revealed in table 6.

TABLE 6

Postoperative Complications

Cardio-Vascular Failure	122
Thrombosis, Embolism	74
Pulmonary Complications	61
Others (Delirium, Psychosis, Convulsions)	30
Total	287
Percent of Total No. of Anesthetics	5.3%

Being interested in the problem of whether or not the length of operation or anesthetic may influence the occurrence of complications, we classified the anesthetics of this survey as to duration of time (Fig. V).

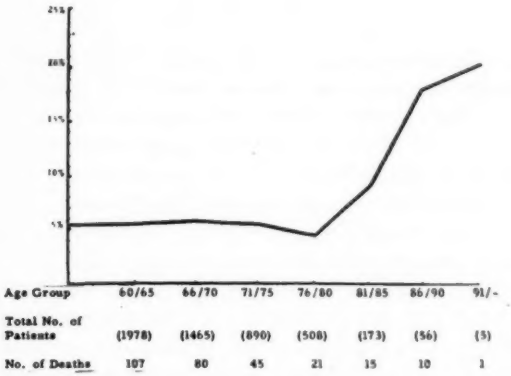

FIG. V. Distribution of 5,450 anesthetics in relation to length of operation.

The average operating time was 88 minutes. Four hundred twenty-two anesthetics, 7.7 percent of the total, lasted more than 3 hours. The longest operation lasted 9 hours.

The incidence of intra- and postoperative complications in relation to length of operation is summarized by figure VI.

One hundred five complications occurred in connection with operations lasting less than one hour, which is 4.8 percent of the total number of operations (2,173 operations); 619 complications occurred in connection with operations lasting more than one hour, which is 18.8 percent of the total number of operations (3,277 operations).

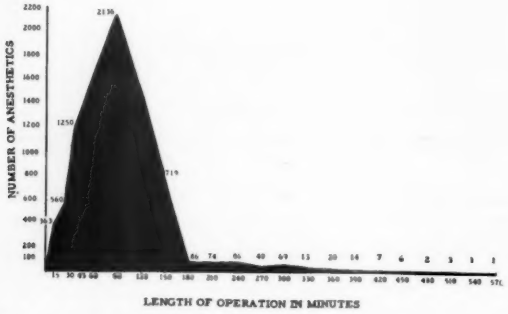

FIG. VI. Occurrence of intra- and postoperative complications in relation to length of operation.

Discussion. The precentage of healthy aged patients, meaning patients free from any systemic disorder, is relatively small (Fig. II). The specific problems of anesthesia for the aged are therefore not so much associated with age itself but with the morbid changes in the old organism due to cardiovascular, pulmonary, renal and other diseases favored by old age.

It is often difficult or impossible to distinguish between an apparently healthy physiological old age with no systemic disorder and senility as the result of old age complicated by a morbid process in one or more of the vital systems. Inasmuch as the healthy old patient should not present any particular anesthetic difficulty, undoubtedly the aged patient with certain systemic diseases may offer quite a few problems and should be considered a poor risk. The anesthetic management of both may not differ at all. They each require the principles of good anesthesia and the skill and knowledge of the well trained anesthetist. There is an additional challenge in dealing with sick aged patients because of the ever existing possibility of their fast deterioration following a cardiovascular or pulmonary failure.

Cardiovascular disturbances are the most frequent preoperative complications. The relatively high number of patients who belong to the group with pulmonary complications indicates that the physiological atrophic degenerative changes of the lung tissue in the aged are often combined with additional morbid processes of inflammatory or non-inflammatory origin.

A severe anemia (hemoglobin below 10 grams) was present in 292 patients. The incidence of cases with a moderate anemia was much more frequent. This seems to be contradictory to other authors (Howell, 1948) who found in the healthy aged person usually no evidence of anemia. Most of the anemic patients of our series suffered coincidently from other diseases such as carcinoma of the urinary bladder, prostate, intestine, bronchus, or tuberculosis of the lungs. In addition to this, they often had focal infections such as chronic cholecystitis, prostatitis or sinusitis. Most of these anemias appear to be secondary in origin.

The choice of the anesthesia method and the selection of the anesthetic agent for elderly patients is a special problem. It should be based on the principle of guaranteeing the utmost safety for the patient. Secondly, the needs and requirements of the surgeon should be recognized. Finally, the anesthetist should be absolutely familiar with all techniques and methods he may use. Anesthesia for aged patients is no place for methods which are not well-established in the anesthetist's repertoire.

In general, we cannot say that we found a special anesthetic drug or particular technique of significant advantage over another. However, we administered cyclopropane in almost all major surgical procedures and especially to the poor risk patients of this series. This gas is especially useful in geriatric anesthesia because of properties such as pleasantness of induction, minimum effect on the coronary blood flow and potency which allows high oxygen content in the anesthetic mixture. The tendency of cyclopropane to cause cardiac arrhythmias in young individuals was not a serious problem in the management of old patients. Arrhythmias incident to increased myocardial irritability were controlled by adding ether to the anesthetic mixture (Allen, *et al.*, 1941). Light cyclopropane anesthesia does not appear to cause significant depression of the myocardium and was well tolerated by elderly patients with advanced heart disease. Deep

anesthesia with cyclopropane or any other agent should always be avoided in patients of this type.

The use of intravenous sodium pentothal was restricted to minor surgical procedures. The drug was usually combined with nitrous-oxide-oxygen in order to utilize the sedative effect of pentothal with the analgesic action of the nitrous oxide. The definite depressant effect of pentothal on the respiratory center is a significant disadvantage. It is frequently necessary to assist the respiration early in order to avoid hypoventilation. The high reflex activity of the vocal cords occasionally associated with the administration of pentothal is of minor significance in the aged because of the reduced autonomic response. Pentothal for the induction of anesthesia was used only for patients showing apprehension or signs of emotional distress. The dosage of pentothal given in these cases did not exceed 3 to 5 cc. of a 2½ percent solution. This amount was enough to allay apprehension and facilitate the application of the mask and the administration of the chosen gas.

More than 90 percent of all the spinal anesthestics given were to patients belonging to the genito-urinary service (trans-urethal and supra-pubic resection of prostate, biopsy of urinary bladder, cystosocopy). The relatively small number of severe complications following this technique (Table 4) justifies the conclusion that low spinal anesthesia for prostatectomies and other genito-urinary procedures is well tolerated by aged patients. In general surgery the use of spinal anesthesia was confined to operations of the lower extremities, the perineum and the lower abdomen. In administering spinal anesthesia above the level of D-10, there is danger of interfering with intercostal muscular activity, which is already weakened by age.

The use of short or longer acting relaxants such as curare or curare-like drugs should be restricted to properly selected patients. As a rule, the musculature of the aged patient is naturally flaccid as compared to that of younger adults. Therefore, there is usually no need of additional relaxation in administering, for example, a satisfactory cyclopropane-ether anesthesia. Tightness of the abdominal musculature is often the sign of hypoxia following an impaired respiratory exchange. In giving curare or curare-like drugs to cover the negative results of an apparently

inadequate technique, one could easily cause a fast deterioration in the patient's condition by increasing the respiratory difficulties and subsequently augmenting hypoxia. The slower breakdown and the delayed elimination of curare in old patients should be another factor to which our attention is attracted if this drug is chosen for use.

Failure of the cardiovascular system was the main factor in the occurrence of intraoperative complications. An unusual blood pressure drop occurred 303 times due to blood loss or neurogenic reflexes. Of the 81 patients who went into profound circulatory distress, severe blood loss was the cause in 44 instances. In many of these cases blood loss and neurogenic trauma seemed to be tolerated for long periods without the appearance of the warning signs of threatening deterioration such as we usually observe in younger patients. When circulatory distress did develop it was sudden in origin and difficult to correct. Patients of advanced age should be followed closely throughout their stay in the operating room with particular attention being paid to the immediate replacement of even the smallest amounts of blood lost during a surgical manipulation. Elderly patients tolerate any degree of hypoxia very poorly and for this reason anesthetic mixtures should always have a high oxygen content.

Provided the fundamental requirements of blood replacement and oxygen supply are considered, the hypofunction of the autonomic nervous system in the aged should not be necessarily detrimental. There is evidence that the often extremely easy and uneventful course of geriatric anesthetic procedures results from this central autonomic weakness. Younger patients may show under similar circumstances more pronounced and often undesirable activity of the central autonomic control. Therefore, in comparing a certain number of anesthesia records of younger patients with an equal number of anesthesia charts of elderly patients a smoother and easier course in the aged in respect to blood pressure level, pulse rate and rhythm and respiratory rate is often revealed.

The number of patients in our series with signs of respiratory impairment during the anesthesia course is relatively small. This might be explainable by the fact that we routinely assist the respiration, particularly in aged patients, as early as necessary to

avoid even a short lasting respiratory impairment. The frequent and generous use of endotracheal tubes and pharyngeal airways to maintain constantly an unobstructed free airway is of extraordinary importance in this connection. The same is true for the repeated use of the suction catheter to remove secretions from pharynx, trachea and bronchial tree during the anesthesia procedure.

The presence of cardiac arrhythmias is considered to be more common in old patients than in younger adults because of the degenerative changes in the aged myocardium with involvement of the conduction system. The occurrence of cardiac arrhythmias during anesthesia is not necessarily a sign of a deterioration of the patient's cardiac reserve but merely the continuation of the usual rhythm. Quite often the origin of suddenly occurring arrhythmias is due to carbon dioxide accummulation, the sudden removal of high CO_2 or an overdose of the anesthetic drug. Sufficient ventilation, adequate oxygen supply and a decrease of the anesthetic agents is the treatment of choice.

The postoperative complications demonstrate similarly to intraoperative disturbances the prevalence of complications which can be related to a failure of the cardiovascular system. Ninety-three patients went into circulatory distress or shock either in the operating room immediately after finishing the surgical procedure or on the ward during the very early postoperative period. We therefore consider this period the most dangerous part of the entire anesthetic procedure. The already reduced autonomic adaptability together with an additional loss of automatic tone induced by the still effective anesthetic agent may be mainly responsible for those pronounced circulatory disturbances after surgery. Hence, we avoid any abrupt and rough handling of the patient in changing his position after termination of the anesthetic and moving him from the operating table to the stretcher. The gradual lowering of the limbs after finishing a procedure done in lithotomy position under spinal anesthesia should be equally important. Once circulatory failure or shock occurs, it is much more difficult to restore the failing circulatory system of the aged patient than that of a young person. The management of postoperative shock depends upon the leading symptoms or the probable origin of the failure. In any event, the

patient should be placed in a slight Trendelenburg position in order to facilitate the venous reflux to the heart and oxygen should be administered immediately. Blood loss shock, of course, needs additional blood or plasma infusions. If these are not immediately available, any other plasma expanders such as polyvinyl-pyrollidon (P.V.P.) or dextram could be used. Elderly patients ofter suffer from dehydration and the danger of overloading their circulatory system is not as great as commonly supposed.

Aged patients are prone to vascular accidents such as thrombosis or embolism in the postoperative course. In our series, thrombosis or embolism which affected either the lung, the coronary arteries or the brain occurred 74 times.

Pulmonary complications do not play a very significant role in our series. Broncho- or lobar pneumonia occurred in only 33

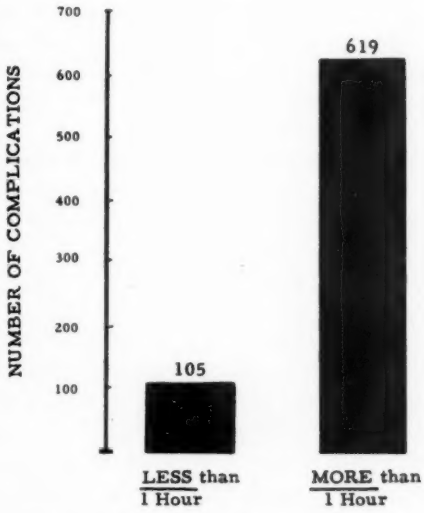

FIG. VII. 270 hospital deaths in relation to duration.

patients. The frequent use of antibiotics combined with the early and repeated cleaning of the trachea and bronchi in the postoperative phase may explain this small number. In spite of the fact that elderly patients easily develop atelectatic processes of the lung following surgery, only 17 cases were observed. Special

attention is directed at all times to the avoidance of any condition which could favor the development of atelectasis in the postoperative period and the ward attendants are particularly trained in using the stir-up regime for this reason. The frequent administration of nasal oxygen and the constant cleaning of the upper respiratory tract are equally important in this connection.

Eighty-five percent of all intra- and postoperative complications occurred in connection with anesthetics lasting longer than 1 hour (Fig. VII). Although most of the major surgical procedures and poor risk cases are included in the group lasting longer than 1 hour, there is evidence, however, that the operating time exceeding 90 minutes favors the occurrence of complications.

Deaths. Two hundred seventy-nine deaths occurred in our series. This was 5.5 percent of the total number of patients. We included all patients receiving a general or spinal anesthetic in connection with a surgical procedure and who died either in the operating room or during hospitalization. Of this number, there were 183 men and 96 women.

We do not intend to report and discuss the deaths of our series in any detail. Lorhan (1949) has previously given an extensive report on this subject and discussed the numerous problems involved. It may be of benefit, however, to give briefly some additional information on the deaths in our series as they occurred in relation to age, physical state, anesthetic methods and agents used and time elapsing from the end of the anesthetic until death. We would like to discuss the different causes of death, considering especially those cases in which anesthesia directly or indirectly could have caused the fatality or contributed to it.

The mortality rate increased with advancing age. There was no significant change in mortality rate from 60 to 75 years of age. The age group 76 to 80 revealed even a decreased mortality, explained by the fact that in this group is included a considerable number of patients belonging to the genito-urinary service who suffered only from a benign prostatic hypertrophy without any other significant ailment. The end of the curve revealing a 20 percent mortality in patients over 90 years of age is not reliable inasmuch as only 5 patients were considered.

The relation between increased anesthetic risk and mortality rate is demonstrated by figure VIII showing the occurrence of deaths in relation to physical state.

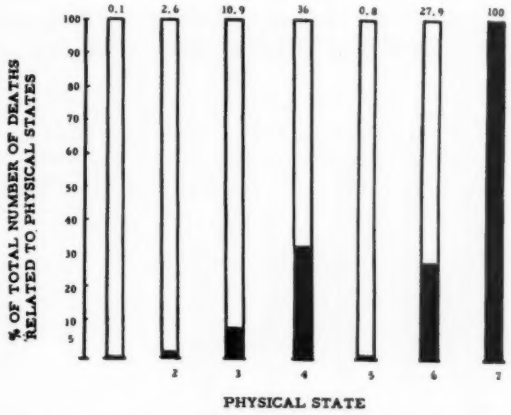

FIG. VIII. 279 hospital deaths in relation to physical state.

A very low percentage of deaths occurred in anesthetic procedures on patients of Physical States 1 and 5. These groups together had a mortality of less than 1 percent. Patients belonging to Physical States 3 and 4 and, of course, Physical State 6, suffering from systemic disorder and undergoing emergency procedures, had the highest incidence of fatality. No patient belonging to Physical State 7 survived to leave the hospital.

Table 7 summarizes the deaths as they occurred in connection with the different agents and methods used.

Not to mislead by this table, we should emphasize that the patient's physical condition and the type of operation required often imperatively dictated the choice of agent and method. That, for example, 8 out of 10 patients receiving only oxygen died is explained by the fact that the greater number of these patients arrived moribund in the operating room and did not require any anesthetic. The same is true of a considerable number of patients who received either a local anesthetic, a regional block or refrigeration anesthesia. The low percentage of deaths following

the intravenous administration of pentothal with and without supplemental gas does not necessarily prove that this drug is especially compatible to geriatric anesthesia because the use of pentothal was mainly confined to minor procedures. A percentage of 6.5 deaths following cyclopropane anesthesia seems to be rather low considering the fact that almost all major and poor risk procedures were done with this gas.

TABLE 7

Anesthetic Agents and Methods Used on 279 Deaths

Anesthetic Agents and Methods	No. of Anesthetics	No. of Deaths	Percent of Deaths
Cyclopropane	2,698	175	6.5
Spinal	1,721	28	1.6
Pentothal	671	21	3.1
N_2O	140	7	5.0
Ether (O.D.)	101	8	7.9
Local and Regional Block	91	29	31.9
Avertin (Rect.)	15	2	13.3
Oxygen	10	8	80.0
Refrigeration	3	1	33.3
Total	5,450	279	5.1

It is difficult to answer the question of whether or not some of the deaths of our series may be directly attributed to the anesthetic agent or method. There is no doubt that in a number of deaths anesthesia played a contributory role. Deaths which occurred either on the operating table or within the first 24 hours postoperatively are especially suspicious in this respect. Figure IX gives the summary of deaths in relation to time elapsing from the end of the anesthetic until death.

Hypoxia which affected the heart muscle was the main cause of the fatalities occurring on the operating table. Two of the 8 patients dying in the operating room expired due to exsanguination and 2 other patients of this group were moribund on arrival.

Thirty-five of the 47 patients dying within 24 hours postoperatively expired due to pre-existing diseases (uremia, diabetic coma, extensive brain tumours with postoperative hemorrhage, peritonitis). Twenty patients of this group belonged to Physical State 7. Three patients went into profound shock shortly after

arriving on the ward and died. All 3 withstood orthopedic procedures fairly well and developed circulatory failure in spite of instant and apparently adequate blood replacement during surgery. In 4 patients the cause of death was a coronary, pulmonary or cerebral embolism. One patient developed an acute thrombosis of the mesenteric artery. Two patients died from congestive heart failure at least partly due to pre-existing heart disease.

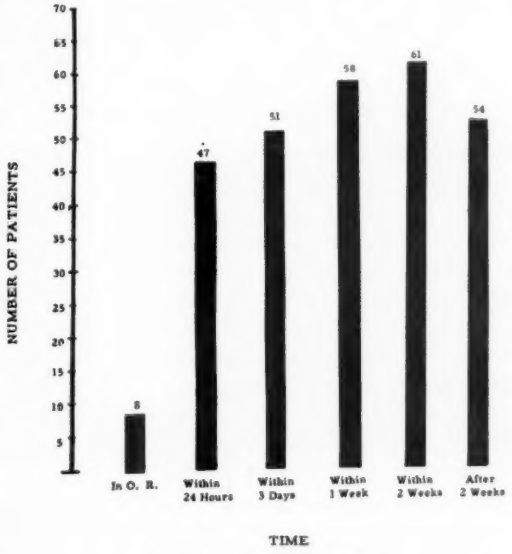

FIG. IX. Time of 279 deaths.

Two patients arrived on the ward in satisfactory condition and died within 4 hours following respiratory difficulties apparently unknown to the ward attendants. It was strongly felt that in both cases the anesthetist was mainly responsible for these fatalities by his failing to insure an unobstructed airway until the patients were more fully reacted.

In some of the fatalities occurring in the operating room or within the first 24 hours after operation, anesthesia may have played a part in causing the catastrophe. Lack of experience and skill on the part of the anesthetist probably attributed much more to these deaths than did the anethesia method or the drug.

The opportunity to keep patients in an especially equipped recovery room under the care of highly trained personnel during the first hours or, exceptionally, for several days probably would have helped to avoid some of these deaths and would have been of benefit to other patients of this series who died in the very early postoperative course.

It would be of interest to compare the number of early postoperative deaths in this series occurring without the facilities of a recovery room with the deaths of a new series for which a recovery room is now available.

Summary

A review of 5,450 anesthetics administered to 5,075 patients ranging from 60 to 103 years of age over a period of 12 years (1942 to 1953) has been presented. The rapid and progressive increase in the number of geriatric anesthetics over the entire period, the distribution of these patients to the different age groups, the importance of evaluating the patient's preoperative physical condition and its significance for grading as to physical state have been discussed. The problems arising in the choice of anesthesia method and anesthetic agent with special consideration to cyclopropane, occurrence of intra- and postoperative complications and their possible connection to the length of anesthetic and operation were also considered.

The deaths were explored as they occurred in relation to age, operative risk, anesthetic management and time elapsing from the end of the anesthetic until death. The importance of a recovery room to reduce early postoperative complications was noted.

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A PSYCHOLOGICAL TESTING PROGRAM FOR ENTERING STUDENTS AT THE UNIVERSITY OF TEXAS SCHOOL OF MEDICINE, GALVESTON*

A Preliminary Report

Molly R. Harrower

For obvious reasons all college administrators are concerned with the problem of the student who fails in his work and flunks out of college. The student who cannot pass the required courses wastes money spent on his education, keeps some other student away, taxes the patience of his teachers, and retards his own discovery of a more appropriate career.

Ideally, a medical school should comprise a student body all of whom learn well, graduate and pass on to become successful doctors who benefit the communities in which they work. In actual fact, despite careful selection by wise and competent experts, we know that there is considerable wastage of personnel each year.

This five-year research program at The University of Texas School of Medicine is directed, therefore, to a better understanding of all psychological factors, intellectual and emotional, which make for success or failure in academic work, and towards the development of new methods of counseling students so as to enable them to make the most of their natural endowments.

This investigation in The University of Texas Medical Branch must be seen as the second phase of studies begun in 1940 in McGill University Medical School. At that time the author developed methods for large scale testing of personality factors, the Group Rorschach method. In this present study, additional group projectives have been used.

During the intervening years, continued research has demonstrated the fact that the same psychological tests which can detect students who are emotionally unable to handle their academic work can also be used to help students understand themselves more fully, and thus make better adjustments to their studies and to life in general.

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The active interest and collaboration of the Executive Director, the Dean of Students, members of the Departments of Student Health and Psychiatry, resulted in the Medical Branch being selected as the most favorable place for this research to be undertaken.

The immediate goals of this research are the following: 1. To alert the Dean of Students and the Student Health Psychiatrist working on the project to the existence of students who appear from the test findings to be seriously disturbed, or to be in such a psychological condition that they may become emotionally disturbed in the near future. 2. To make available on twenty-four hours' notice a detailed report on any individual student who may seek psychiatric help voluntarily, or be referred to the psychiatrist through some other source. 3. To make a survey of the psychological characteristics of the entering class as a whole, so that certain aspects of personality as reflected in the test scores may be correlated with academic or professional success or failure.

The long range goals of this research relate to detailed follow up investigations such as ultimately may allow us to predict confidently the type of student who may be expected to succeed in the profession of medicine. Studies at other universities make possible a comparison of medical students with other student populations. Long range goals also include perfecting techniques of effectively counseling students on a short term basis with regard to specific personal and academic difficulties (projective and tutorial counseling).

Methods. The students are briefed as to the general purposes of the testing program, its research implications and benefits which they, themselves, may derive from it during the orientation program on the opening day of the semester.

The class then meets in two separate sections, with approximately eighty students in each group, for a three-hour period beginning at noon and running into the late afternoon. At the beginning of each testing period the examiner again briefs the students more specifically on the general purposes of the investigation, maintaining an informal and friendly atmosphere throughout the actual administration of the test. Special booklets

have been devised so that all the test material for each student is kept together.

Careful consideration has gone into the selection of the eight tests which comprise this present "test battery." The battery includes the Wechsler-Bellevue and projective tests aimed at estimating various aspects of a student's personality, such as his drive, his emotional stability, his freedom from tension or anxiety, his motivation, and his steadfastness of purpose. The projective tests used are the Rorschach, the Szondi, the Most Unpleasant Concept, the Figure Drawings and other graphic techniques, part of the Thematic Apperception test and the Sentence Completion test.

The initial scoring and evaluation of the eight tests on each of the 160 students is a time-consuming performance, and occupies the principal investigator on a full time basis for the next two-and-a-half months. After each of the individual tests has been assessed and scored, a "personality profile" is made up for each student, and a general personality assessment written as the result of the information derived from the various tests.

For further validation of the findings after they have been assessed by the principal investigator, certain individual tests are submitted to assistant psychologists who make estimates in terms of these single test procedures. The findings are then interrelated.

All students whose test findings appear to show an unusual amount of psychological stress, or excess psychological tension, are "screened" and their names are forwarded to the Dean of Students for his information.

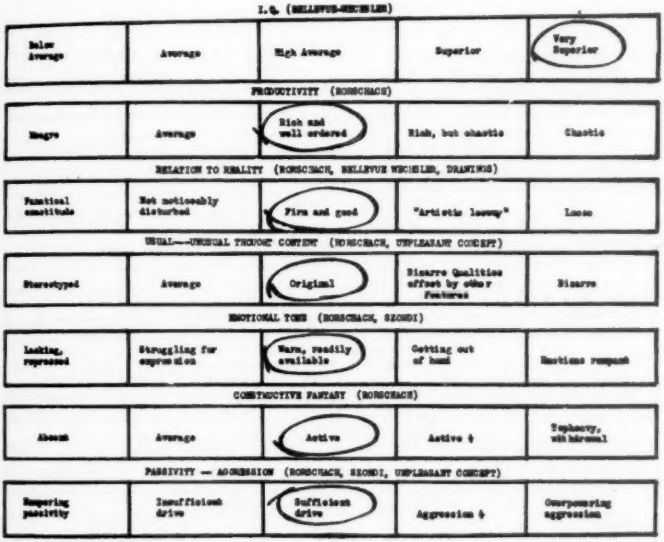
The information on any given student is available in two different forms. It is available as a brief summary as, for example, is illustrated in charts 1 and 2. It is also available in terms of a detailed report, running to four or five pages, in which the test findings are assessed with reference to any particular difficulty that the student may be having. This information is sent at the request of the Dean of Students to the psychiatrist working on the project.

When it seems advisable, information on a student may be given by the psychological investigator to any member of the faculty genuinely concerned about a student's progress, and

seeking to devise ways to aid him in his work. Conferences have been held concerning specific students with members of the faculty, and a helpful exchange of information has taken place in regard to the student both as seen from the tests and as seen from his work.

CHART 1

Summary of Test Findings of a Student Showing Superior Personality Characteristics



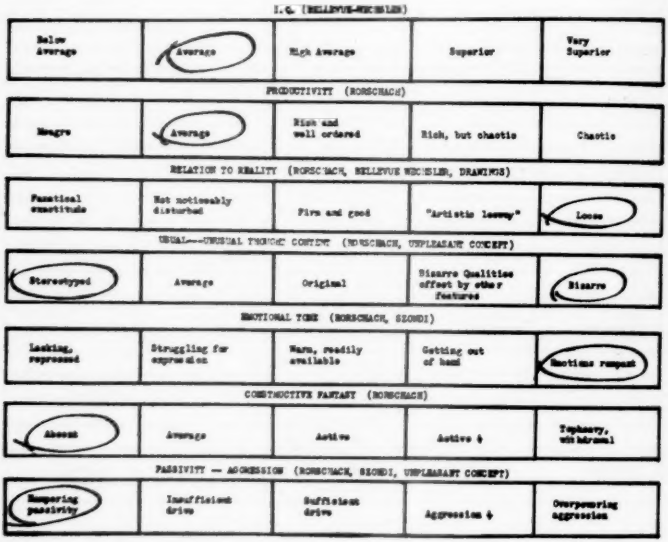
It is also possible to develop a better understanding af academic failure or personality difficulties by the use of counseling sessions with parents and students individually, sessions which throw light on various aspects of the student's difficulties.

Utilization of Test Findings. The unique aspect of this screening program has been the fact that psychiatric help has been provided for students who wish assistance in minor or acute personal problems. There is also available to the students a counseling session in which academic difficulties may be seen in the light of their psychological strengths and weaknesses as

reflected in the tests. The test material is, therefore, used both directly and indirectly—directly, in the face-to-face discussion with the student and indirectly, in the form of a report which can be made available to the psychiatrist within twenty-four hours in order to facilitate and accelerate his treatment of any student who may turn to him for help.

CHART 2

Summary of Test Findings of a Student Seriously Disturbed at the Time of Testing



One of the major points of emphasis in presenting this program to the students themselves has been to make them aware of the advantages which may accrue from greater self-knowledge. A study of the self, it has been emphasized, should not be seen as the morbid preoccupation of individuals who are mentally ill but as a legitimate source of study by those who wish to develop their psychological resources to the fullest. Students have met both individually and in groups, taking advantage of discussions with psychiatric and psychological advisors.

Over and above the help which may be given to any individual student there is a vast accumulation of material relating to the scores of the students on each of the eight tests. In any one test there are many variables which can be assessed with regard to the academic success or failure. It is not expected that any one finding will be the answer to such a complex question, but only by a search for patterns of interrelated findings can we hope to make reliable and helpful predictions in regard to any specific student.

Preliminary Results. Surprising as it may seem at first glance, it is not necessarily the more intelligent students who do the best work. Otherwise expressed, when a student has a personality problem this problem may render his otherwise excellent intelligence inadequate for dealing with academic work at that time.

In order to facilitate tabulation each student was given a code number, those with the highest I.Q.s receiving the numbers 1, 2, etc., those with the lowest being numbered down to 161.

Chart 3 will show that a student may fail as easily in academic work if he has a high I.Q., in terms of the group as a whole, as when his I.Q. is low. Those individuals with squares surrounding their code numbers were those failing in one or more subjects at the end of the first semester. Students whose code numbers have been circled are those who have failed so badly in academic subjects that they have been forced to withdraw, or are those whose personality problems introduced difficulties, such that they could no longer cope with the medical curriculum.

As can be seen from Chart 3* many students with lower I.Q.s are successfully handling their academic work. This fact means that with good motivation and emotional stability a student may

*Statistical Note by J. Allen Scott, Professor of Statistics and Epidemiology.

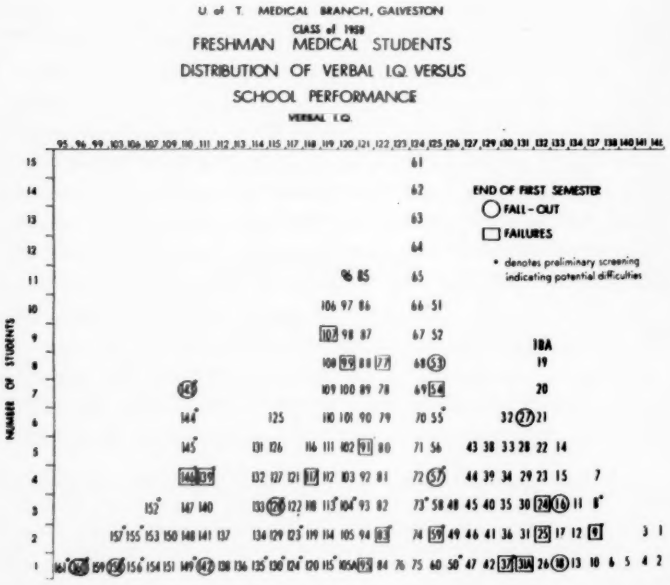
The verbal I.Q. scores of chart 3 present a reasonably normal distribution The distribution of fall-outs and failures does not differ from that of the passing students, the means and variances of the two groups showing no statistically significant difference. The distribution of the Rorschach productivity scores in chart 4 is, on the other hand, essentially a log-normal distribution. On the average the fall-outs and failures have lower scores than the passing students. These two groups differ significantly with respect to their arithmetic and geometric means at approximately the one per cent level of significance.

compensate for less spectacular achievements in the intellectual sphere alone.

Both these findings, namely, that high intelligence is no guaranty of success and that the lower intelligence students can also function well, shows us that in picking the best group of medical students something other than their straight academic and intellectual achievement must be looked for.

In an attempt to isolate factors other than intelligence we selected, amongst many possible variables, productivity on the group Rorschach. Chart 4 shows that if productivity is used as a criterion instead of the I.Q.s. we come much closer to predicting who will have difficulties and who will do well academically. Four of the five students who give fewer than ten responses

CHART 3

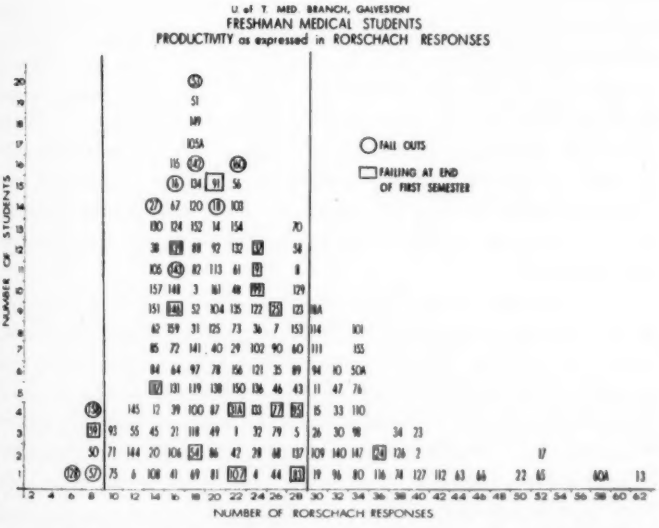


in the Rorschach have either had to withdraw from college (the circles) or are now doing poor work (the squares). At the other extreme we find that all students who have thirty or more re-

sponses will be found to be doing well academically, with only one individual in the group who has a failure in one subject.

Provided these results are confirmed as tests are given to subsequent entering classes, it may be that we have an important clue. In the event of a screening program being inaugurated prior to a student's entry into medical school, this information could be used in combination with the initial interview at the time when the student turns in his application. Assessing the number of responses is a variable which is simple and quick to calculate, so that large numbers of records could be assessed in this preliminary way by technicians even without the psychological assessment the psychologist must give the record as a whole. While this would not mean that a student who could not reach a score of ten responses would automatically be rejected, it might mean that it would be wise to give more careful scrutiny

CHART 4


U. of T. MED. BRANCH, GALVESTON
FRESHMAN MEDICAL STUDENTS
PRODUCTIVITY as expressed in RORSCHACH RESPONSES
FAIL OUTS
FAILING AT END OF FIRST SEMESTER
NUMBER OF STUDENTS
NUMBER OF RORSCHACH RESPONSES

to possible personality difficulties prior to his acceptance. Conversely, it might be suggested that the selection committee could safely accept those students whose scores in this particular test were thirty or above.

From the research angle we want to know not only who fails but what failure means. When a student cannot study, what is the reason? When a student does bad work, what does "bad work" really mean? One aspect of this research investigation is to look into the test findings of those who are doing poorly in an attempt to find out why. For example, we may ask has the student an unusually poor memory? Or is his difficulty that he does not understand clearly what is said to him? Is his problem that he cannot follow instructions? Is the difficulty such that he attempts to make vague generalizations before he has really assimilated the facts?

These and many other similar questions can be asked of the test material itself. With answers to these questions we hope to develop techniques for helping those who are having specific difficulties at certain stages in their medical career, and thereby preventing unnecessary fall-outs which are due to relatively minor errors in study habits. One of our most striking findings is the difference that is found in the relationship of generalizing to analytic thinking, as reflected in the Rorschach, between twenty students who are failing, on the one hand, and twenty students with A and B grade averages, on the other. Chart 5 shows the difference in the W to D ratio on the Rorschach test in these two groups. The A students closely approximate the expected norms in their W to D ratio, while the failing students show a reversal, a marked reduction in the expected number of D responses.

It is clear that once a student's difficulty is detected it is possible to bring it to his attention and, by pointing to his test scores, show him in what direction some of his difficulties may lie. For example, a student, unaware of why he is doing badly in anatomy, may be shown that his score on the memory test falls below what may be expected of him, in view of his other achievements. It is, therefore, suggested that he become aware of this relatively weak link in the chain of his psychological equipment, bending his efforts to overcome it. It is particularly interesting to see how a student's attitude towards his problem can change when it becomes something concrete that he can tackle. He may have had a vague "hunch" about some trouble, but not have been

able to formulate it to himself or to others, so that he could do nothing about it.

It might well be asked, "Is there a type of personality who gravitates to the study of medicine, and do certain personality types make better research workers?" Such questions we will

CHART 5

W–D Ratios, for A Students and Failing Students (Composite Scores)

	W	D	d	dds		W	D	d	dds
14	....	....	....	....	14	....	....	....	....
13	....	xx	....	....	13	....	....	....	....
		xx							
12	....	xx	....	....	12	....	....	....	....
		xx							
11	....	xx	....	....	11	....	....	....	....
		xx							
10	....	xx	....	....	10	....	....	....	....
		xx							
9	....	xx	....	....	9	xx			
		xx				xx			
8	xx	xx	....	....	8	xx			
	xx	xx				xx			
7	xx	xx	....	....	7	xx			
	xx	xx				xx			
6	xx	xx	....	....	6	xx	xx	....	....
	xx	xx				xx	xx		
5	xx	xx	....	....	5	xx	xx	....	....
	xx	xx				xx	xx		
4	xx	xx	....	xx	4	xx	xx	....	....
	xx	xx		xx		xx	xx		
3	xx	xx	....	xx	3	xx	xx	....	....
	xx	xx		xx		xx	xx		
2	xx	xx	xx	xx	2	xx	xx	....	xx
	xx	xx	xx	xx		xx	xx	xx	xx
1	xx	xx	xx	xx	1	xx	xx	xx	xx
	20 "A" Students					20 "Failures"			

hope to answer from the kind of statistical material that is provided from the test findings. To give one very simple example, it is possible to estimate any individual in terms both of the strength of his imagination, on the one hand, and his need to work closely with other individuals, on the other. In popular terminology, the "withdrawn, introverted" type and the "out-

CHART 6

INTROVERSION-EXTROVERSION RATIOS EXPRESSED GRAPHICALLY

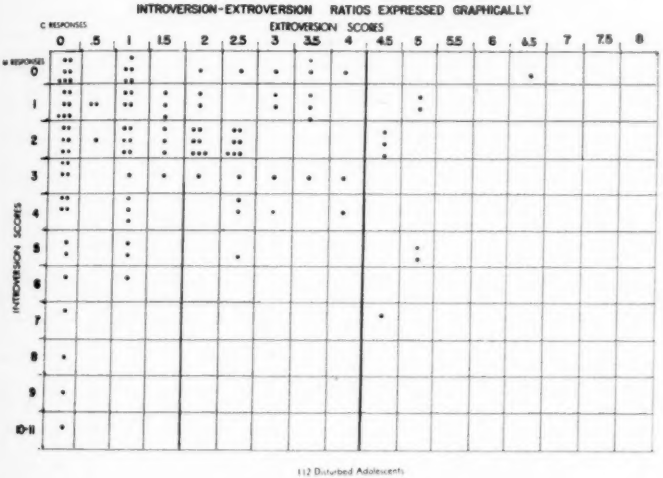
C RESPONSES

EXTROVERSION SCORES

INTROVERSION SCORES / M RESPONSES	0	.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8
0	131 126			89	157	149											94
1	150 89 79	118 87	72 58	82	64 114 108 115	132 105 92	59		139				129				
2	134 108 180 158 145 158 37	3 160 143	41 121 93	12 96 7 35	73 70	68 135 113	104 101	124		54	91						
3	131 127 145	6	97 161 117 56 68 28 73 89 77 67	105 48 138 53	46 61 19	18	40 153 144	12 60A		111						11	
4	95 87	153 69 113 82	58 146 48 50	78 106 134 108	20 21 52 118 31 109 87	1 110	14 36A	47									
5	46	36 148 44	60 51 122 88 132 4	155 154 147	26	140 131 127	76 16		70 25								
6	8 83	63 84A 38 91	32 66	90 100	126 43 84		23				119						
7	105A	74	36 136		137	30		80			84	17					112
8					65	98											
9		49		89 50A			9	10									
10-11							34 32		8								

going, extroverted" type. The attached chart shows various positions in which an individual may be placed in terms of these two personality characteristics. We may ask, do the most intense research workers show up to be those with the greatest amount of imagination? Is the successful doctor with a large country practice some one who enjoys himself in outgoing activity? We may ask what the professional future will hold, for instance, for students number 94, 112, 2 and 49 who are a-typical in terms

CHART 7


INTROVERSION-EXTROVERSION RATIOS EXPRESSED GRAPHICALLY
EXTROVERSION SCORES
C RESPONSES
0 .5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
INTROVERSION SCORES
0 1 2 3 4 5 6 7 8 9 10-11
112 Disturbed Adolescents

of the general population, and have widely different extroversion-introversion scores.

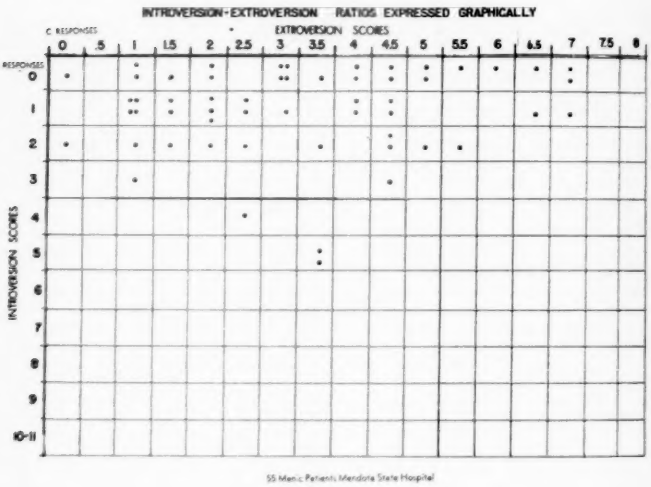
This student population may now be compared with two abnormal groups: Chart 7 shows a group of 112 disturbed adolescents who were referred for therapy. Chart 8 has the scores from 55 manic patients in a state hospital. The scores on these two abnormal groups differ markedly one from the other, and may both be contrasted with that of the "normal" population.

We feel that the place for an accurate psychological screening program is unquestionably before a student is admitted to medical school, rather than during his first year. At this point, a con-

siderable financial saving could be expected. Our long range goal, therefore, as mentioned previously, is so to perfect this type of study that it may safely be employed as *one* of several screening devices which may take place prior to the student's acceptance as a medical student.

This is a five-year project, and no hasty judgments or conclusions will be reached. We are encouraged, however, by some

CHART 8


INTROVERSION-EXTROVERSION RATIOS EXPRESSED GRAPHICALLY
C RESPONSES
EXTROVERSION SCORES
0 .5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
M RESPONSES
INTROVERSION SCORES
0 1 2 3 4 5 6 7 8 9 10-11
55 Manic Patients Mendota State Hospital

of our findings at the end of the first semester as to the reliability of the tests to detect a certain percentage of those who "cannot make the grade."

We are also greatly encouraged by the attitudes of the students to the opportunity which is provided for them to gain an increased understanding of themselves through the means of psychological testing and tutorial counseling, and a second report dealing with these aspects of our program will be published shortly.

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IDEALS FOR A COMMUNITY HEALTH LIBRARY*

CHAUNCEY D. LEAKE

Proudly you are dedicating this day a magnificent new community health library. You have ample justification, members of the Houston Academy of Medicine, both for pride and for an abiding sense of responsibility in maintaining this great new center for regional health service, made possible through the enlightened benefaction of the Honorable Jesse H. Jones.

Your pride and responsibility are humbly shared to some extent by me. Over a decade ago the late generous and enthusiastic Doctor William Bertner discussed often with me appropriate arrangements for a central health service library for Houston, in connection with the extensive new health center being planned. It was exciting to think then that an ideal health library could one day be developed here. Now you have it, and it is equally exciting to be participating in its dedication to ever increasing use as a community health asset.

How thrilling it must be to you to realize that you have here a unique economic treasure, capable merely by continual intelligent operation not only of steadily increasing dividends in good health for everyone in the area, but also of steady growth of the principal in capital potential of providing more good health as well. Here is really a vigorous expanding company worth the closest attention of the shrewd and prudent investor, for even this day the good health of the people of Houston is worth more to them individually and collectively than all the oil and other riches which have made this city so world renowned. Whatever may promote that good health is an investment of prime priority, and here in this community health library you are making precisely such an investment. May it always remain at the top of the blue chip list in your portfolio of securities.

A health library is a new concept. It is part of the changing emphasis in the purposes and goals of training, research and service to humanity in respect to health. As usual the magnanimous

* Address delivered by the Executive Director of The University of Texas Medical Branch, Galveston, at the dedication of the Jesse H. Jones Medical Library, Houston, Texas, September 9, 1954.

and far-sighted leadership of the health professions is bringing new goals for optimum health for the people into ever sharper focus. A great library such as this is one of the chief tools in such focusing of emphasis, and one of the major instruments in trying to reach the broad goal of better health for everyone.

New Goals in Community Health Effort. Humanity's approach to the problem of disease has gradually changed with snowballing results. First there was long-lasting primitive fear of disease, with superstitious efforts to control it, some of which unfortunately remain with us. There was then the tedious gradual accumulation of insight and experience through centuries of torturous trial by a growing self-teaching professional group especially concerned with handling disease by diagnosis and treatment, when it appeared. Thirdly, there presently developed a slow gathering of specific and precise verifiable knowledge about the causes and control of disease, resulting in a shift of emphasis at the beginning of this century toward the prevention of disease. Now we are finding that we know so much about the causes and control of disease, and that we have become so skilled in handling it when it does show up, that we can attend to what is really of paramount importance to us all, the promotion of optimum health for each of us. Forward moving workers in the health field will increasingly keep the goal in mind of trying to get good health for all. In this worthy effort a library such as this may really help.

Health libraries have played a major role in the changing attitudes of people toward disease. While primitive fear of disease is still with us, and while traditional methods of handling disease result in physicians sitting back to wait for disease to come along, the bringing together and the analysis of scientific knowledge about disease has caused a much more aggressive attitude currently in an attempt to prevent disease. Unfortunately, our economic imagination lags behind our scientific knowledge, so that doctors cannot yet make a satisfactory living by practicing on the basis of preventing disease. When doctors learn that people are willing to pay to keep healthy, maybe real efforts can develop toward the goal of promoting optimum health. In the special field of dentistry some progress has been made in this direction.

The Growth of Health Libraries. Even in primitive times

medicine men found that the complications of experience about the complexities of disease were too much for memory. Records about the diagnosis and treatment of disease began to be kept as early as the fourth millenium B.C. Ancient Egyptian physicians had well organized teaching texts, both in surgery, as in the Edwin Smith Surgical Papyrus, surviving from an original of 3500 B.C., and in medicine, as the Ebers Papyrus, destroyed in Leipzig in World War II, but written around 1800 B.C. These venerable teaching guides, maybe from some old health temple library, were organized along modern enough lines to include case reports, and etiological theory in addition to considerations of diagnosis, prognosis and treatment. The old Egyptian general practitioners had practical formularies such as those surviving in the Hearst Medical Papyrus at the University of California, and the Berlin Medical Papyrus. There were even texts for specialists such as the Petrie Papyrus in London for gynecology, and the Chester Beatty Papyrus in London for proctology. There is thus clear evidence for health teaching efforts in old Egypt, clustered around health temples perhaps, where there must have been collections of records and texts. Similar great collections of medical recipes and procedures were made and preserved on clay tablets by the ancient Sumerians.

Here we have an opportunity through the research facilities of this great new institution to collect and study similar compilations of primitive recipes for handling disease as made by the ancient Aztecs in Mexico or by other native peoples in this area. How shocking to realize that as we destroyed the natives and their cultures so well adapted to the great geographic variations of Texas, we also lost forever their tradition of local plant and mineral remedies which they may have found by long and patient trial in their search for food.

The great health temples at Cos, Cnidus, Pergamon, and Epidaurus, in ancient Greece, were training centers for health personnel, and must have had extensive libraries for teaching use and reference. Records of observations were made at these centers, such as preserved in the remarkable Hippocratic collection from the school at Cos. Here was an extraordinary health library. It became one of the most important sources for the development of rational medicine in our culture. Even now its philosophical

impact is marked, both with regard to the support of high ethical standards of practice, and in a naturalistic and non-mystical approach to the handling of disease. The value of this library is attested from the care with which its remains have been preserved through two and a half millennia.

In the ancient Chinese and Hindu cultures, vast collections of health writings were made and preserved for teaching. Unfortunately, in these instances the ancient texts became stereotyped, were followed blindly as curiosity languished, and additions or changes were frowned upon. In this stagnation of scientific interest in health, a static condition for treating disease developed, so that no advance was possible for centuries. Nevertheless, the Chinese are reputed to have evolved an economic method for payment for the preservation of health.

As Greek culture was absorbed by the Romans, many health libraries must have developed throughout the Roman world. Individual physicians as Galen, seem to have had large collections of manuscripts, resulting in many references to previous writers in their own reports. In Alexandria a huge library of manuscripts was assembled, and it became a center for scholarly study particularly in medicine. Unfortunately, this great library was gradually dissipated through pillage and fire in religious riots.

Even during what is called the Dark Ages in Europe, Graeco-Roman health writings were preserved in monasteries, and were translated for the rapidly expanding Arab culture to the east. Here they were studied and treasured in the Mohammedan empire, later to be translated into Latin for the benefit of teaching centers which were gradually developing in Italy and western Europe. At Salerno, the main health center of the 13th Century, there must have been a considerable library. Manuscripts were preserved there in the old Greek, as well as from translations into Latin from Arabic sources. With commentary and exposition by the Salernatin teachers, the example was set for university medical students in the new teaching centers of Europe, such as Bologna, Padua, Paris, Oxford, Cambridge, Montpellier, Basel, and Prague.

Libraries of medical books became an essential part of the working tools of individual physicians and of health training centers as soon as printing began in the 15th Century, and mass

production of books was made possible. Even so health books were precious. They were carefully bound and often chained to reading desks in the libraries. Usually the libraries were locked, and were permitted to be used only by fully accredited scholars. Only too often these mediaeval traditions shackle too many of our own modern health libraries. Nevertheless, the tradition of the high value of a health book is well worth maintaining.

Libraries became the special interest of medical organizations and academies as they developed in Europe in the 17th Century. Outstanding was the library of the Royal College of Physicians in London. The library of the Royal College of Surgeons became a center for postgraduate teaching. However, these libraries maintained the mediaeval traditions, and only individuals with the most critically screened qualifications were allowed to use the books. Often the volumes remained locked in chains. Unhappily this situation still exists.

Under these influences the great collections of books and manuscripts on health affairs in teaching centers in Europe became relative static. Physicians began again to collect their own records, and to compare results in meetings and discussion. With specialized interest, special libraries for separate health disciplines developed. During the 19th Century most of the medical, pharmacy, and other specialized health schools in the Germanic sphere of influence developed separate institutes with separate libraries. The result was great duplication. Ordinary students had little access to these specialized libraries for reference use of readings, and as a result had to purchase the books needed for personal studies. The example of the brilliant clinical teachers in Paris, London, and Edinburgh, and in Vienna, Berlin, Leipzig and Munich, stimulated the more eager students, among them many Americans, to accumulate helpful personal libraries. In the 19th Century it was still possible for an enthusiastic physician to have a personal library reasonably satisfactory for the total health knowledge available at the time. Three to five thousand volumes might cover the entire range of medical contributions for personal use, including rapidly growing special periodicals. Doctor Ashbel Smith (1805–1886), pioneer Texas physician, and brilliant and medical educational leader in early Texas, must have had a library of this sort. What happened to

it? Similarly, Doctor Greensville Dowell (1822–1881), who started the medical journals and medical schools of early Texas, had a similar library. Where have its books gone? Here is something for the historical section of this library to investigate.

During the 19th Century there was rapid mushrooming of medical schools in the United States. With the speedy expansion of the country, and with poor communication, this was necessary in order to provide medical care under frontier conditions. Under these circumstances there could only be poor library facilities. Attempts were made to develop student use libraries at Harvard, at the College of Physicians and Surgeons in New York, at Pennsylvania, at the University of Virginia, at Tulane, and even at the frontier school in Transylvania. One of the more cultured members of the faculty would be given several thousand dollars for a trip to Europe and the purchase of books for the school library. Most of such efforts were unsatisfactory. However, as medical education in the United States was stabilized at the beginning of this century, with the elimination of proprietary and ill-equipped schools, a standard part of the teaching program was centered around the medical library.

Some medical schools developed exceptional libraries, such as the Lane Library at Stanford Medical School in San Francisco. Similarly the Welch Memorial Library at Johns Hopkins Medical School has become outstanding, through the guidance of Doctor Sanford Larkey, and this has been the case also at the Yale Medical School under the brilliant inspiration of Doctor John Fulton. In some of the major cities of the country, local academies of medicine have developed extensive libraries which function as teaching and reference centers for the health sciences in the area. Very significant in this service has been the example by the New York Academy of Medicine, the Philadelphia College of Physicians, the Boston edical Library, the Library of the Cleveland Academy of Medicine, the libraries of the St. Louis County Medical Society and of the Los Angeles County Medical Society, and the John Crerar Library in Chicago.

We are fortunate in this country in having the greatest health library in existence, the superb Armed Forces Medical Library starting humbly from a tiny collection of 228 volumes in 1840, but receiving its greatest impetus for outstanding development

from the vigorous efforts of that amazing medical genius, John Shaw Billings (1838–1913). This has now become a national health library of over a million volumes, whose catalog alone is a reliable index of the health wisdom of mankind.

Medical libraries rose slowly in Texas. That intellectual spark-plug, Ashbel Smith, proposed in 1848 that the State support a central medical library. In writing to the Legislature on behalf of the Medical and Surgical Society of Galveston, to plead his standards for an organized medical profession in Texas, he said:

> Among the means of acquiring knowledge a good and ample Library holds a high place. This must embrace many works whose cost would surpass the *means* of most of the members of the profession, besides the numerous valuable periodicals, the subscription to all of which would be an *intolerable* tax to any *single* practicing physician or surgeon.[1]

The Legislature after proposing a bill to implement Doctor Smith's request, amended it to the table. In 1851 Ashbel Smith wrote:

> We have no medical organization in our State; nor is there much prospect of any change. Each member of the faculty is a separate independency and sometimes adopts a sort of armed neutrality system.[2]

Now a century later the medical and other health professions of Texas are well organized and able by their collective energies to support many fine health libraries as at San Antonio, Austin, Dallas, and Fort Worth. The University of Texas Medical Branch Library in Galveston is now the most extensive teaching and research health library in the Southwest, and serves the area as a whole, rather than the local community. Here, in Houston, however, is the most sumptuously housed and, because of its community service potentials, the most promising health library for effective achievement. When your centennial is celebrated let it be hoped that your full promise will have been realized.

The Health Team Concept. With the expansion of modern health endeavor, and with the goal of promoting optimum health for everyone, the health team is expanding enormously. In recognition of the trend, some universities such as Ohio State, are wise enough to designate a combination of these activities as "The

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[1] P. I. Nixon, *Medical Story of Early Texas*, San Antonio, 1946, page 466.
[2] Ashbel Smith, "On the Climate and Diseases of Texas," *Fenner's Southern Medical Report*, 2: 458, 1851.

Health Center." The health library becomes the central focal point for the training, research, and service effort, not only for medicine, but for all the auxiliaries to the health team. The modern health library must contain full reference service for all aspects of medicine, dentistry, public health, nursing, veterinary medicine, pharmacy, clinical laboratory technology, radiation technology, clinical psychology, physical therapy, occupational therapy, sociology and social service, dietetics, medical record librarianship, and hospital administration. Indeed the alert health librarian must now be prepared to anticipate the need of intelligent laymen, especially lawyers, in health affairs, and have material ready which may bear on all sorts of phases of the current health effort, including economics, philosophy, history, geography, politics, sociology and psychology.

Your new community health library will pay particular attention to promoting increasing interest in psychological factors associated with health. Outstanding in Texas is its Mental Health Program, so well sponsored by the Hogg Foundation for Mental Hygiene. We will increasingly need mental poise and equilibrium with control of our emotional reactions if we are to satisfactorily maintain our freedom and the advantages of our way of life. The most subtle undermining of our ways of living can occur psychologically, particularly by any yielding to authoritarian pressure.

It is significant that psychology is actively participating in health team work. An excellent example of the importance of psychological factors in rehabilitation is given in the program of the M. D. Anderson Hospital. Recently The University of Texas pioneered a conference on Medical and Psychological Team Work in the Care of the Chronically Ill. Such teamwork is an important item in the adaptations that health personnel may make that are conducive to better health in a community. Case records, reports of conferences and discussions, and formal contributions on psychological matters relating to health may well become one of the most important features of your community health library.

Whatever you may do to promote teamwork among the health professions will be reflected in general betterment of community health. Team work is essential in any health activity,

since no single individual, however wise or vigorous, can either know or do alone for community health, what can so much better and quicked be accomplished by group effort and teamwork among well-trained health personnel. Psychological factors are paramount in the successful working of health teams. In this community health library you may bring together information on these often intangible factors, which may make it possible for the health workers in this area to set a worthy example in efficient community health service. Somewhere civilian health workers should demonstrate that they can surpass in economical and efficient teamwork the achievements so often proudly accomplished under military stress. Maybe here is the place to try, through this community health library.

The Reference Function of a Community Health Library. A community health library may be a most helpful reference center, from which all the people in the area may derive aid in the promotion of their individual and community health. As in any other library, a community health library should maintain basic reference sources in the humanities, in literature, in aesthetics, in ethics and logic, in philosophy, and of course in the broad scientific fields of mathematics, chemistry, physics and biology. There should be plenty of historical reference information, and basic data on economics, sociology, anthropology, and even geography and geology. Health seems to be dependent on the intelligent application of the interplay and coordination of all knowledge.

These basic reference sources are usually provided in a general university library. If a community health library is to be promoted at any distance from alarge university center, it is essential that this basic material be available for ready reference. The classic magnanimity of medical and health workers will countenance whatever duplication of material may be necessary for the promotion of the morale of the growing health team.

A community health library is primarily designed for convenience of use, as you have so well done here. Thanks to the effective cooperation of your health leaders in this community, and with the skill of an outstanding architect, you have provided a building of marked utility, comfort, and attractiveness. How wise you have been to furnish so excellent an auditorium for use

in connection with your community health interest! In your delightful lounging rooms you have made it possible for health workers throughout the community to get together for pleasant informal interchange of ideas, from which there is bound to come increasing inspiration and stimulation to apply whatever knowledge you may be accumulating here to the best advantage and to the good health of all the people of this great and blossoming area.

Since all workers in the health field are mature and responsible individuals, with serious goals in mind, the community health library can well be arranged so that users may find quickly and easily the material they want. Increasingly difficult is the problem of providing satisfactory indices and abstract and review articles. The tremendous snowballing growth of health knowledge has resulted in such a vast assemblage of factual material, that the very problem of finding out what we do know in health matters is becoming a strangling one. Let there be plenty of pencils and notebooks handy for ready reference, not only with your indices, abstract and review journals, but also in your shelves and cubicles where you arrange your monographs and periodicals.

It may be possible some day to provide microfilm packages of various collected articles dealing with different health matters, so that at touch of a switch, the pages may be thrown across the screen for rapid review for the workers who may be concerned. It may even be possible to make such collections of significant data that they may be available for long range television, so that life saving information can be flashed from this great center of reference to hospitals and clinics everywhere in this area. Certainly the well known and highly respected ingenuity and initiative of Houstonians can make such health service possible.

The Goals of a Community Health Library. Now that you have such a superb building, such a splendid collection of books, and such expert and skilled librarians to manage it for you, what are you going to do with it all? It thus becomes pertinent to examine goals and purposes. Wise we would all be if we were to keep in mind our purposes! This is always of paramount importance for health workers. If health workers are going to remain in primitive fear of disease, then little advance can be made

toward an effective goal. If we are to sit back and wait for disease to come along, we do not have a very stimulating purpose. On the other hand, if we do embrace the prime purpose of attempting to promote the good health of all the people in this community, then a great community health library such as this may indeed have a worthy purpose of which all who may be associated with it may be proud.

The basic purposes of this Jesse H. Jones Community Health Library are the same as any other health library: (1) to collect and preserve the knowledge and experience which has been accumulated on the cause and management of disease, and on the promotion of health; (2) to add to this accumulation of knowledge by the recorder experience of current workers, including formal publications, but also remembering to preserve manuscripts and notes of progress; (3) to make readily available to all who wish to use this accumulation of knowledge and experience on health and disease for the promotion of health or for the management and control of disease, and (4) to maintain professional standards and ideals, and to provide goodwill service to all who come to seek help in health matters. However, you may well show your famed ingenuity and spirit of achievement in finding some specific and unique purposes for your own community health library.

The activities of The University of Texas School of Dentistry strongly suggest the importance of promoting as close liaison between the medical and dental and related professions as may be possible through the services of the community health library. Similarly the interests of the M. D. Anderson Hospital suggest that special efforts be made through the community health library for the fullest possible library service in relation to tumors. An exceptional opportunity is available to you in connection with poliomyelitis. Similarly you have challenging openings for community health library service in regard to the control of drug addiction, and in the promotion of optimum handling of traffic casualties. One of your greatest contributions might come in devising workable principles for economic and efficient health service. You might promote special collections in medical psychology. One of your greatest contributions could well be special col-

lections for health in old age, the problems of which may too soon affect all of us.

Some of these special interests may seem local, with reference to your community only, but if the services which are developed are effective, they may have world-wide significance. Geographic and political boundaries have no meaning in relation to disease, and through the promotion of good health, men of goodwill might greatly relieve some of the serious world tensions under which we all labor. You may wisely consider the free publication and world-wide distribution of significant contributions to the promotion of health which may come from your own members.

In a new community health library such as this, it is increasingly hard to decide what to preserve of the accumulated wisdom of the ages on health and disease. Careful choice is needed in getting old classics in original editions. Here members of the Academy may show their generosity by providing such treasures for inspiration and delight. The mere burden of collecting, binding, cataloging, and arranging the current health literature, of something more than 7,000 periodicals annually from all over the world, is a terrific job in itself. If this community health library is really to serve its broad purpose, an attempt must be made to choose wisely among this vast amount of material, and to preserve carefully that which seems to have significant interest.

Research and Teaching in a Community Health Library. This community health library will be in itself a marked stimulus to research. Research is of fundamental importance in the health effort. In the practice of medicine or of dentistry, each patient who comes to the clinic or hospital is really a special research problem. It is the task of the physician or dentist to be curious regarding the situation in that patient, to study that patient thoroughly, to find out what is the matter with that patient, and then to devise the most satisfactory way of handling that patient's problem. Even the problem of promoting optimum health for any given individual is a research effort. It is therefore important that the research attitude be fostered and encouraged in any health center. Thelibrary ca nbe the main driving force in this research endeavor.

The chief function of this magnificent community health li-

brary will be in teaching and training. It will provide a convenient, comfortable, and pleasant central study place for students in all the health fields, and for those of the general public who may want to use it. Perhaps one day you can provide health book-mobiles, with health exhibits, which may carry health information directly to the people. You may indeed use the facilities of this fine building for health exhibit purposes. Whatever you may do through your collections of books, periodicals, pictures, and physical objects, to increase the appreciation of people generally in the promotion of health, will be worth your while.

You will see to it that the different health institutions in this area are coordinated with this central health library. You can offer excellent aid in advice and in actual books and pamphlets or reprints in the development of departmental and hospital libraries for immediate reference and use. You can encourage rapid distribution of material from this central health library to the worker in a special laboratory or clinic where the material may immediately be needed. With an interest in the teaching programs in the different institutions in the health center, you can arrange appropriate displays of illustrative material, to supplement the training program, and you can make readily available pertinent reference sources for the use of the students. Reference lists may be distributed or posted in a way to correlate with the subject matter of the different courses. This would be a great help to the teaching program in the hospitals and schools of the center.

What fun you may have in organizing seminars for review of significant current health developments. Most urgently needed in the modern health endeavor is the critical review effort. There is so much material available, that it is hard to handle unless it is digested, analyzed, correlated, and synthesized into generalities that can be readily appreciated and applied. Such an effort could be a prime function of seminar groups which might be organized under the auspices of this great health library. You might indeed from these seminar efforts develop an important health review. This might appear periodically, and be one of the most significant contributions that you could maintain.

Your historical collections are certain to become an increasing source of pride and pleasure to you. Here may be collected the

archives related to the health effort in this community for future study and interest. Here also may be arranged exhibits from time to time to give a cultural background to the various health fields who use this library. You will make an effort to obtain at least one copy of an early edition of an outstanding work of every great medical contributor. This will help in orientation in the health discipline. It will give a sense of proportion and value in regard to the slow accumulation of our current power in health affairs. We all know best what we learn from the beginning. Your archives of reprints, contributions and manuscripts of staff members from the health center will provide an important record of achievement, and may help the teaching program by aiding students in knowing at first-hand what their teachers actually do. Such a collection is a considerable morale builder for the professional staff members.

It is amazing how fecund a community health library can be. Try the experiment of putting an old vellum bound incunabulum, like this one by an unknown Roman printer of 1499 of Albertus Magnus, "The Secrets of Women" (titillating title!), next to a copy of some current masterpiece, like this one on tissue culture of your good neighbor, Charles M. Pomerat, and you may be surprised how all the glorious classics of medical achievement may appear between them. When good brains are bred with good books, genius may be conceived and come to term.

L'Envoi. Members of the Houston Academy of Medicine, you have now dedicated the Jesse H. Jones Medical Library to community health service. It is a lofty responsiblity which you have assumed, and you have fittingly indicated your seriousness of purpose, with the generosity of the Honorable Jesse H. Jones, by planning and erecting this superb building. This structure is the covering and protection for the intellectual treasures within. Here indeed may be the "brain," the coordinating intellectual center for all the manifold expressions of community health endeavor. Like the brain of a person, this collective brain is sturdily protected against injury, and the dignified face that goes with it reflects the serenity of the wisdom within. May you always have reason to be proud of the way in which the intricate brain of your community health program, this library will work.

THE IDEALS OF SCIENCE IN RELATION TO NATIONAL SECURITY

CHAUNCEY D. LEAKE*

It is not easy to talk about science and security. The very terms are becoming emotionally charged with varying significance for different people. It is difficult to control feelings in considering the matter, and it is hard to keep away from personalities.

It is proposed, however, to discuss the situation in generalities, without reference to personalities, and yet in as open and frank a way as possible. Many implications are made of a very serious nature to specific individuals. Case histories of scientists who are most involved are available, and have been exhaustively analyzed, even in the popular press. Many other scientists, however, are confused, discouraged, or resentful, over anonymous denial of security clearance to serve their country when they recognize its needs. There is a clash between the usual ideals of scientists and the authoritarian enforcement of security regulations in our country.

The ideals of scientists are not generally understood. In this age the applications of scientific theory largely determine the character of our culture. Yet, the aims, the spirit, and the methods of scientists are scarcely appreciated even by those people who may benefit most from them.

For scientists, science is a way of life; for politicians science is an instrument of policy; for the mass of people, science is something they don't know much about, which they vaguely fear and distrust, but which seems to help them live longer, more comfortably, and certainly produces a lot of fascinating gadgets.

Scientific endeavor suggests by its very nature that its results are to be used for the benefit of humanity. Unfortunately some men may apply scientific developments to evil purposes. It is this possibility which alarms scientists, for they would like to assist in the democratic way of open discussion, in determining what their work is to be used for.

When scientists find that their scientific discoveries may be

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used to injure or destroy people or to harm or wipe out any of the beautiful or good things of the spirit which human beings have created, then they are distressed. Under these circumstances scientists may become obsessed with a sense of guilt, and their scientific judgment falters in an emotional confusion which they desperately seek to avoid. This is to say simply that scientists are human, and would profit by self knowledge, in depth, as Lawrence Kubie emphasizes.

Scientists in this country assume that their scientific ideals are similar to those held by scientists in other countries. In the past this assumption has been reasonably well justified. Under any form of authoritarian pressure, however, scientists anywhere may be forced to compromise their ideals for political, financial, or biological expediency. By "biological expediency" is meant all that may be involved in the risk of living.

Authoritarian pressure on scientists may be exerted nationally by creeping bureaucratic control, by extra-legal "hearings" and similar refined methods of torture, or by economic factors for which scientists themselves may be responsible. Such authoritarian threats to science appear even in our country when security procedures contravene the basic American principles of the assumption of innocence until proven guilty and of rejecting *ex post facto* judgments.

Authoritarian pressure may become evident in the policies of industries toward employees, and even in the regulations and rules of University Boards of Trustees regarding faculty members. Against such pressures, scientists, who generally are peacefully inclined, tend to compromise their professed ideals and standards. Such compromises, tending toward sterile conformity, are really not in the best interests of a society which may attempt to force them.

When representatives of the people abuse their positions by defiling human dignity in their orgies of accusations and in their lust for authoritarian power, they fail to promote loyalty to what they may represent or to increase the security they may claim to desire. We simply cannot enforce the democratic way of American life by lining up those we suspect and purging them from us. These methods fail because loyalty and security are matters within each one's own personality. Loyalty and security

are psychological concepts, voluntarily developed in each individual by certain favorable environmental factors and by certain basic personality traits which are capable of being psychologically conditioned.

It is my purpose to discuss the aim, the spirit, and the methods of scientists in connection with their scientific endeavor. This will provide a background for consideration of factors involved in national loyalty and national security.

It is my hope to indicate that loyalty depends on a vast complex of interpersonal relations involving common standards and ideals. For us in the United States this means loyalty to the democratic way of life as exemplified in the history and traditions of the American people.

It is further my hope to emphasize the importance of developing our concept of national internal security toward the maximizing of scientific gains rather than toward the minimizing of losses. It will be indicated that national security depends upon the collective individual loyalty, good sense, and deportment of citizens, including scientists, which can be promoted and encouraged in a positive way from within better than by force or fear exerted from without.

If there is to be genuine loyalty and security, without hypocritical evasion or lip-service, it must be promoted and encouraged in a positive way from within.The real loyalties of a person cannot be obtained by force or fear exerted from the outside. Fighting loyalties to principles for which a nation stands are gained by voluntary free-will from within each person, as a result of inner conviction.

The people of this country seem to have dedicated themselves to the preservation of responsible freedoms. As Leonard Woolf indicates, authoritarian conditioning from within and authoritarian ruthlessness from outside, are continuing threats to our freedoms, but can inevitably yield to our increasing psychological knowledge, if we learn to use that knowledge well. Scientists may indeed be able one day to show how democracy among men may flourish without fear of authoritarian control.

The Aim, Spirit and Method of Science

From Aristotle to Einstein, from Hippocrates to Schweitzer, certain men, curious about themselves and their environment,

have developed concepts of the aims, the spirit, and the methods of their special endeavor. These notions have crystallized into the abstraction called "science." This abstraction and the applications of it largely make our culture what it now is.

Our predominantly scientific culture transcends the artificial and arbitrary boundaries of political units, thus sometimes arousing conflicting claims to the loyalties of intelligent persons with curiosity and interest in the world and the people in it. It is characteristic of the better scientists continually to be extending the horizons of their interest from their personal selves through their families, through their social and professional groups, through their national associations, to the world at large.

American scientists, with the material benefits of the results of their activities achieved within a democratic frame-work, can honestly work for the accomplishment of the same satisfactions for all people, in the same kind of a pattern. This they find is similar to the Christian idea.

The business of scientists is to find the "truth" about ourselves and the world about us. The problem is to reach agreement upon "truth." The Platonic concept of ideas still troubles us, and semantics has not yet satisfactorily resolved the difficulty. Even sicentists themselves fall into the trap of thinking of "science" as something real in itself.

In scientific work "truth," as a symbol, means an intellectually coherent but admittedly tentative explanation of some aspect of ourselves and our environment, which has been objectively demonstrated and agreed upon by those who have investigated it. What we mean by the "truth" is the opinion agreed to by all who investigate the matter, and it is the object represented in this opinion that is the "real."

One of our most distinguished scientists, once President of the American Association for the Advancement of Science, and of the American Philosophical Society, the late Edwin Grant Conklin, identified the aim of science with that of religion, to know the "truth" about ourselves and the universe, with confidence that even unwelcome "truth" is better than cherished error. This places an ethical aspect on scientific endeavor. This is also the goal of those techniques devoted to self-knowledge, such as psy-

cho-analysis and psycho-therapy. Truth gained in this self-correcting attitude is conducive to that mental health which we all need so much these days.

According to Conklin, the spirit of science implies not only freedom to hold and to express any view for which there is rational evidence, but also to recognize that knowledge of ourselves and our environment is incomplete and subject to revision. Further, it is emphasized that there is no legitimate compulsion to belief, beyond the voluntary acceptance of demonstrably rational evidence. The only authority which scientists can recognize is the voluntary agreement, among those who study a matter, which may be reached on the basis of objective controlled data and the logical deductions derived therefrom.

The scientific method of arriving at "truth" is now well defined in theory and practice. On the one hand, a scientist may try to build a coherent ideal structure within the rigid limitations of logical consistency, by means of experimental reasoning. With this idealization, some details of the universe about us may be found to correspond. This is the way of mathematics.

On the other hand, one may observe and describe as accurately as possible one's self and one's environment, or some aspect thereof. A tentative explanation may then be suggested of ways by which an individual or the environment of that person may operate with respect to the particular factor under examination. The validity of this tentative explanation may then be tested by experiment, which seeks to isolate and control specific factors. Conclusions are reached in accordance with the results of such experiments. On this evidence, voluntary agreement may be reached regarding the validity and significance involved therein. This is the procedure followed in the natural sciences.

Knowledge of ourselves and of our world acquired by these methods has vastly altered our way of living. We are discovering that this sort of scientific knowledge makes it possible for us to add significantly to the broad and lasting satisfactions of all of us. This suggests again that scientific endeavor has moral value.

It is also clear that scientific knowledge is acquired by cooperative endeavor, involving scientists in many places and times. Scientific "truth" is independent of political boundaries. Unfortunately, politicians in different countries may make vastly different uses of the same scientific "truth."

In many instances the choice of the use to which scientific "truth" may be put, may be an individual one. Scientific "truth" may be used for good and beneficial purposes for all humanity, or for evil and detrimental purposes to individuals or to humanity as a whole.

The uses to which scientific "truth" are put would wisely seem to be best determined by broad agreement among all those who have access to such "truth." For it may reasonably be assumed that all people wish to benefit themselves. Under these circumstances a compromise between the interests of different individuals or of different groups might work to the best advantage for all. In spite of the truculence of publicity statements by various national leaders, this seems to be the reasonable approach to problems of nuclear energy, as offered by President Eisenhower through the United Nations Organization.

The Problems of National Security in Relation to Scientific Work

The logical developments of scientific knowledge regarding nuclear energy can be utilized by any group of people who have the technological ability to do so. The basic scientific facts on nuclear energy are now known to scientists all over the world.

Under these circumstances it becomes vastly important to put as much attention on the proposition of acquiring more knowledge about such nuclear energy, and of utilizing it, as to waste valuable time in fooling ourselves that a potential enemy may not realize the significance of published scientific information.

The theory of poker as a game shows that one really has a better chance to win if one goes on the assumption that one's opponent knows just as much of what one holds as one knows one's self. This procedure gives freedom for the utilization of logic and energy to defeat the opponent on the basis of what the situation actually is. Victory in warfare is almost as much a matter of psychology as it is of weapons and manpower.

We must, of course, to survive, use discretion, avoid discussion or comment on possible developments of nuclear energy for warfare or other purposes, and be discreet in expressions of generalities on these matters, or in conversation on specific details of them, with anyone not well known to be trustworthy, honest, and thoroughly reliable. Above all, let the Internal Security Act be wisely administered.

The Board of Directors of the American Association for the Advancement of Science has stated the point of view of American scientists in a clear and succinct way:

> Those charged with safeguarding the United States have sought to minimize the danger of internal subversion through the screening of government employees and persons having access to classified information. This program is necessary, but it poses a serious dilemma: the more completely we succeed in reducing the danger that information now in our possession may leak to a potential enemy, the more risk we run of interfering with scientific progress and of reducing the technological superiority and the moral and physical strength upon which victory in the ultimate test would depend. The inherent dangers of this dilemma can be lessened and our strength enhanced by changing our basic concept of internal security from one that attempts almost exclusively to minimize our losses to one that places greatly increased emphasis on maximizing our gains.

The difficulty with the security regulations in the United States is that no one knows exactly what they are. Conformity can generally be obtained among any group of people who are working toward the same ends, if the purposes, aims and procedures are known and understood.

Not even government workers, let alone scientists who may be concerned in governmental affairs, know what is expected of them, in conformity with security regulations. Security clearance is usually denied on the basis of what is called "derrogatory information." What does this mean? Who makes this judgment and how?

The difficulty is compounded when the security regulations change. There was great need for security during World War II. Nevertheless at that time it was desirable to have a certain amount of interchange of ideas, personnel, and equipment, with our allies, particularly Russia. Of scientific and humanitarian interest, for example, was the American Soviet Medical Society. This published a number of volumes of a journal, giving English translation of significant current Russian contributions in medicine and health. The Society was a professional one, and in no way has any subversive activity been associated with it. Now, however, what American scientist or physician who might have joined the Society from the best purposes possible, can be sure that his membership is not used against him?

One of the most serious aspects of the current security situation in the United States is the anonymous character of charges.

A reputable scientist eager to contribute what he can, either as part of a home team, or as a visitor invited from abroad, may suddenly find that he is denied security clearance. He has no idea what the specific charges are, he knows nothing of who has made them, and he is given no opportunity to examine the validity of the charges or the reliability of those who have made them.

Since withdrawal of security clearance implies a reflection on the character of the scientist concerned, there is obviously serious injustice if there is no chance at defense. Unfortunately no systematic method has been devised by which such problems can be handled in an open, frank and above-board manner.

The result is that marked psychological anxiety is developing among American scientists. On the one hand, there are many who are reluctant to have anything to do with scientific matters involving security or with government-supported research. On the other hand there are those who become very loud in their loyalty, and who, in their reaction against anxiety, get aggressive, in assuming an authoritarian position and in making themselves conspicuous in their self-righteousness.

The matter is of the utmost seriousness in connection with atomic energy. Developments in this field will enormously affect all of us. None of us seem yet to realize what is implied in the power of the hydrogen bomb.

Nevertheless, atomic energy is expensive. At the present time only the United States and the USSR have the funds for extensive scientific and technological development in this field. People would be wise to be honest in comparing the situation between the Russians and the United States in regard to atomic energy.

It has long been assumed that the Russians, as a result of authoritarian control of the ideology of Russian scientists, could never make great scientific advances or technological progress. This assumption is false. Russian scientists are now publishing excellent scientific material of much practical consequence, and they are showing great engineering skill. Authoritarian control of the ideologies of scientists, as the Russians learned from the Lysenko fiasco, does not produce the goods.

Russian technological development, in spite of all obstacles, is much better than Americans think. While the output of scientists and engineers in the United States has been dropping, the

number of scientists and engineers in Russia has been steadily increasing. It may very well be that the Russians can surpass the Americans in the development of atomic energy either for war or peace by sheer probability of effectiveness from greater numbers of skilled young scientists and engineers. American youngsters seem to be getting soft in their reluctance to tackle the stern disciplines of modern science, especially in mathematics, physics, and chemistry.

Of utmost concern are the security regulations of the United States in regard to this matter. The belief is widespread among Americans that the Russians are progressing only by stealing "secrets." No scientific "secrets" are involved. The physics of atomic energy is now universal knowledge. The important matter is technical know-how.

In modern industrial methods, particularly those associated with atomic energy, technical know-how is a complex mixture of scientific knowledge, engineering skill and procedure, and technological art which cannot be satisfactorily described even in the most detailed publication or written program. In order to acquire the technical know-how of such a complicated industrial program as that which involves atomic energy, it is necessary for teams of highly skilled workers to be sent for months or years to those who want to acquire the know-how in order to get the process running.

This is well recognized in industry, where for many much simpler processes, such as chemical or metallurgical manufacturing, teams of skilled workers have to try for months or even years to get groups in other countries to do the job well. To believe that such know-how can be handed over to an enemy by some spy whispering some words on a street corner, or by passing over secret documents, is simply nonsense.

However we like it, it would be wise for us to realize that the Russians are probably the equal to the Americans in knowledge of atomic energy technology either for peace or war. Actually the essential scientific knowledge regarding the hydrogen bomb was developed earlier by the Russians than by the Americans.

Sir Francis Simon of the Clarendon Laboratory at Oxford, warns us about the dangers of our extraordinary preoccupation with spying and security in regard to atomic energy. Our diffi-

culty stems from our general public ignorance of the way in which scientists work.

Sir Francis, like the Board of Directors of the American Association for the Advancement of Science, emphasizes the importance of promoting the development of scientific ideas. Ideas develop through honest criticism. Scientific progress is greatly retarded if there is any interference with the free exchange of scientific ideas. This free discussion of scientific ideas, with frank honest criticism, breeds new scientific ideas and better technical methods. To block such free interchange of scientific ideas, in the effort to get full security among scientists, would result in almost full sterility.

Sir Francis proposes that a realistic understanding of the way in which scientists work would help in getting a balance between free exchange of information and ideas in order to promote scientific development on the one hand, and in preventing important and easily transferred information from reaching an enemy on the other hand. While security regulations are necessary, they can be developed sensibly and with a minimum of interference with scientific and technological activity among the scientists concerned.

We may as well frankly admit that Russia's power in the atomic field is now dependent on the size and quality of her own scientific and technological effort, and quite independent of us. The Russians probably realize that our security hysteria is really helpful to them, and thus deliberately do their appropriate part in maintaining it.

The situation, as Sir Francis concludes, is a very depressing one. We are faced with vigorous and aggressive Soviet expansion. Instead of making every effort to maintain scientific and technological superiority, we dissipate our efforts and stimulate our emotional confusion by intensive and authoritarian security regulations and procedures. The result is that we inhibit, irritate, and antagonize our scientists, who are the very ones who are most essential for our effective progress and defense.

Summary

We cannot escape the conclusion that the authoritarian methods in which our security regulations are enforced, are detrimental to our best scientific interests and indeed to our very

safety. Two reasons for this conclusion are paramount: (1) our security program gives us a false sense of strength and safety, which is dangerous in the face of an aggressive, determined, and scientifically capable enemy, and (2) our security procedures inhibit the very work of our scientists and technologists on which our safety and welfare depends.

It is tragic to consider the effects of the false sense of security which is given the American people by authoritarian methods of attempting to preserve "secrets" of scientific work in this country. The opportunity for demagogues to exploit the situation is particularly dangerous, since it arouses so many emotional misunderstandings among the people regarding the ideals and standards of scientists.

If our scientists are to lose their standards and ideals and if our people fail to keep their sense of value and judgment, we are sunk, and me might as well prepare for a coming age of barbarism. Vannevar Bush clearly indicated what scientists and men of good-will can do if they preserve their freedom and their strength. no matter what the challenge. He also warned clearly of the wreckage facing us if we alienate our scientists. Such alienation could indeed force scientists to become a "lonely crowd" in a waning civilization.

The current situation calls for an intensive vigorous campaign on the part of American scientists to explain their standards and ideals, so that there may be broad public understanding and appreciation of what scientists try to do for human welfare. The more verifiable knowledge we can get about ourselves and our environment, the better we should be able to control ourselves and our environment for our benefit and welfare.

The responsible officials of the United States government would be wise if they were to make a thorough effort to understand the full implications of scientific endeavor in this country, so that it may be maintained in a free, democratic, and wholesome state, which will really help to preserve and extend those liberties and privileges which we profess to treasure.

It is the duty of those who are concerned with the philosophical significance of science to continue steadfastly to tell the people what science is about, and what the standards of scientists are. This is something beyond the usual sort of popularization of

science. It calls for much patience and tact. If it can succeed in reducing the threat of authoritarian control of American scientists, it will be worth everything that it may take.

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METRAZOL THERAPY WITH SUCCINYLCHOLINE–CHEMOPLEXY*

NEVILLE MURRAY

One of the earliest reports of physiodynamic therapy was made by Weickhardt in 1798. He recommended the giving of camphor-in-oil to the point of producing vertigo and epileptic fits to patients who were suffering from emotional disorders. A novel about contemporary French psychiatry in the 1800's contained a description of convulsive treatments during which patients were contorted into various bizarre attitudes and such was their intensity that muscles were torn from their insertions and bones were snapped like match sticks. It is little wonder that the treatment fell into disuse over the following one hundred and fifty years.

In 1934 Meduna, at that time in Budapest, recommended the treatment of schizophrenic psychoses by the induction of convulsions with the intramuscular injection of a 25 percent solution of camphor-in-oil. The treatment was suggested on the basis of the dubious observation that epilepsy was rare in schizophrenia and the subsequent assertion that there might be a biological antagonism between schizophrenia and epilepsy. Meduna claimed to produce 91 percent of "remissions" which included recovery, social recovery and improvement in those cases of less than one years duration. Camphor was replaced by other drugs which could be administered intravenously and produce a more rapid induction of convulsion. These included such drugs as cardiazol, triazol and picrotoxin. (The term *cardiazol* is synonymous with Metrazol or pentylene-tetrazol.) Finally, Cerletti and Bini in Italy produced, in 1937, therapeutic convulsions by passing an electric current through two electrodes placed on the forehead and this form of induction superceded the chemical type as a safer, more convenient and less painful method of convulsive therapy.

It is at this point that electro-shock therapy almost completely

* Read at the Mid-Continental Psychiatric Association, Little Rock, Arkansas, September, 1954.

replaced convulsive therapy by chemical induction. It was thought grand mal seizure, no matter how caused, was the therapeutic end point. It was, therefore, only just that the most expedient method to attain this therapeutic goal should be the one most commonly employed. However, subsequent pharmacological investigation has revealed that there are certain qualitative differences in convulsive phenomena. Although one grand mal seizure may clinically appear to be very much like another, no matter how caused, the physico-chemical changes within the brain substance may not always parallel the outward clinical manifestations.

Even in the clinical field it has been noted that individual cases have responded to Metrazol-convulsive therapy which have proven unresponsive to electro-convulsive therapy or other types of physiodynamic treatment. Such are the undesirable side effects of Metrazol therapy that it has hereto only been employed with reluctance on comparatively few occasions. Textbooks have stated that electro-shock can do anything that Metrazol-shock can do with far less inconvenience and clinical observations to the contrary have largely been ignored.

The intention of this paper is to outline a technique for the administration of Metrazol-shock therapy designed to eliminate those undesirable side effects by using the muscle relaxant drug Succinylcholine Dichloride (Anectine). A substance which has already improved the technique of electro-shock therapy. Secondly, to discuss some of the salient features which justify the more frequent use of this type of chemical convulsion therapy which I have chosen to term "Chemoplexy."

The undesirable effects of Metrazol therapy as originally performed may be listed as follows: (1) prodromal feelings of anxiety and impending death; (2) severity of convulsive spasm causing fracture in as many as 40 percent of cases, as reported by Polatin; (3) the production of *status epilepticus;* and (4) post-convulsive discomfort characterized by vomiting, malaise, and muscular discomfort.

The following technique involves measure designed to obviate these undesirable effects and is notable for ease and simplicity of administration.

A careful physical examination is prerequisite to treatment. It

is to be noted that cardiac disease is not an absolute contra-indication. Each case, as in all physio-dynamic therapy, should be judged on individual merit. The patient should be treated on an empty stomach. Pre-medication should be administered one hour before the time of treatment in the form of intramuscular injection of atropine (1/75 gr. to 1/150 gr. according to individual tolerance.) The atropine serves a multiple purpose. First, to decrease the salivary and mucous secretions which might be aspirated during the period of areflexia, to decrease or diminish the gastric secretions, to reduce the tone of involuntary muscle and abolish spasmodic contractions causing pain and vomiting. Secondly by action on the heart and circulation in blocking any undue stimulation of the medullary Vagal center. Finally, it tends to decrease the convulsive threshold of the cortex and to stimulate the medullary respiratory center so aiding counter-action against apnoea.

The patient is then placed in the supine position and an intravenous injection of Succinylcholine Dichloride in doses of 10 to 20 mg. (½ to 1 c.c.) is made rapidly through a 20-gauge needle. The needle is left in place and a syringe containing 10 cc. of a 10 percent solution of Metrazol is substituted immediately after the first injection of relaxant.

Both Metrazol and Succinylcholine are compatible in the same syringe but the time taken to achieve adequate muscular relaxation with Anectine is 15 to 20 seconds . . . some 8 to 10 seconds longer than the time usually taken for convulsion to follow to intravenous administration of Metrazol. By having an assistant compress an alcohol sponge proximal to the end of the needle in the vein during the change of syringes excessive leakage of blood can easily be overcome. If the operators have some difficulty in changing syringes on the needle two operators may be employed, one on each arm and through separate venepuncture a second injection can be given on the contralateral side. The exact time for the administration of Metrazol is indicated when the fasciculations, notably in the small muscles of the face, characteristic of disorganized neuro-muscular transmission are first seen. These fasciculations are not painful and if the injection of Succinylcholine is given slowly their production is minimal, however,

their presence is desirable since they herald the imminent onset of muscular relaxation suitable to the purpose.

The second injection, Metrazol, is given rapidly and is followed in 8 or 10 seconds by a grand mal seizure which is discrete in onset and comfortably modified. There is no initial tonic spasm and there only remain softened clonic movements primarily localized in the extremities. The duration of these convulsive phenomena is somewhat more lengthy than those seen in the electrically induced type. Spontaneous respiration begins some 40 to 45 seconds after cessation. The central stimulating effect of the atropine and Metrazol compensates for the peripherally induced apnoea of the curare-like substance.

Two cases have occurred where convulsive phenomena persisted after the onset of the spontaneous respiration for some three or four minutes. In both cases they were successfully terminated by immediate administration of an electro-convulsive dose. Ten ccs. of Metrazol has invariably produced loss of consciousness of the petit-mal type if not grand mal seizure. If the convulsive threshold is not reached on the initial administration of this dose of 10 cc. of Metrazol 15 to 20 cc. may be given without toxic effect on subsequent occasions. The patient, thus treated, regains consciousness with 10 to 15 minutes following treatment and one is impressed clinically by the marked confusion and restlessness compared with that observed following electrically induced convulsion. This period of confusion and restlessness is short lived and within 10 to 15 minutes the patient will fall asleep to awaken with little or no recollection of the treatment situation far less subjective discomfort. If post-treatment vomiting does occur it is notably responsive to chlorpromazine. Post-shock headache is easily controlled by the administration of aspirin by mouth.

This type treatment is particularly suited to combination with deep-shock insulin therapy. Georgi concluded that there is a close similarity between the physiological changes induced by insulin and those produced by Metrazol, the former being a slow motion picture of the latter.

Physiological changes occurring during Metrazol convulsions consist of a pre-convulsive change in blood sugar level which is reversed during the inter-convulsive stage. At the same time

there is a fall in the potassium level during both pre- and interconvulsive stages with a simultaneous increase in calcium ions. These changes point to an increased permeability of the cellular membranes and a consequent increase of ionic interchange between the cell and its surroundings. It is this physico-chemical reorganization which is believed to exercise a therapeutic effect.

At the end of the prescribed period of insulin coma interruption is carried out by intravenous injection of 50 cc. of 50 percent glucose and the needle is left in situ. As the patient begins to show evidence of positive response to environmental stimuli the syringe containing Succinylcholine is substituted and the two-stage injection outlined previously is carried out.

This type of therapy would appear to bring about a more profound functional reorganization in the individual than that seen following electro-shock therapy. Memory disturbance seems to be more severe as well as other evidences of confusion; but it is worthy of note that in a recent discussion of the proceedings of the British Medical Association section on Neuro-Psychiatry, July, 1954, Doctor R. S. Ferguson reported less E.E.G. disturbance following Metrazol-convulsive therapy than seen in electro-shock therapy.

Frequency of administration of treatment is usually restricted to one or two per week. The average course of treatments is from six to ten sessions.

In the fifty cases we have treated at The University of Texas Medical Branch and subsequently in private practice the results have been sufficiently encouraging to justify wide-spread use of this type therapy. Recovery figures of statistical validity are not available. In many cases treatments were combined with other physio-dynamic measures; however, the general clinical impression was that in many instances there were significant responses only after the induction of Metrazol therapy.

One case, a woman in the middle sixties, had a severe schizo-affective disorder with profound depressive components, had shown no response to twelve E.S.T.'s and was considered, by virtue of her cardiovascular condition, to be a poor risk for a course of deep-insulin therapy. This patient showed a remarkable response to a course of ten *chemoplexy* treatments which were given without complications. There were no clinical fractures in

the series. However, these results were not proven by X-ray findings. Other complications were minor and appeared no more inconvenient than those seen in electro-convulsive therapy cases. Patients were questioned closely about their subjective feelings with this type of treatment and in most cases there was an apparent absence of discomfort. In several cases electro-induced and chemically-induced convulsions have been alternated with the result that patients were unable to differentiate between the two.

There were no fatalities. A comparison of death rates was given by Ebaugh in 1943 and is worthy of mention. The respective rates were: .6 percent with insulin; .5 percent with E.S.T.; and .1 percent with Metrazol.

The reasons for paradoxical responses to Metrazol-convulsive therapy as opposed to electro-convulsive therapy are not known, but pharmacological research has noted differences in the preconvulsive processes occurring in *chemoplexy* as opposed to electroplexy. Chance (1951) showed by an ingenious method of assessing brain metabolite concentration, notably the glycogen increment pattern produced by various convulsive drugs that Metrazol produced glycogen increases in the cortex, a somewhat lesser increment in the medulla and almost as marked an increase in the mid-brain and spinal cord as in the cortex. These resulting patterns are interpreted as indicating the distribution of nervous activity in the brain arising from drug stimulation. A parallel tracing is shown using the drug picrotoxin. The author further states that this medullary and cortical influence of these two drugs indicate their clinical suitability as antidotes for barbiturate poisoning.

The administration of barbiturates prior to Metrazol-convulsive therapy so raises the convulsive threshold that grand mal seizure cannot occur. One patient who after ½ gram of sodium amytal injected intravenously received 20 cc. of Metrazol without effect, but on subsequent occasions convulsed adequately on 10 cc. of Metrazol given without any barbiturates. Electro-shock convulsion threshold, conversely, is not influenced by barbiturate narcosis. Barbiturates have been reported by some to decrease the convulsive threshold in electro-shock therapy.

Further presumptive evidence is provided by Chance (1951). He studied the anti-convulsive action of histamine and vasodilators in Metrazol and electro-shock therapy in man. Chance showed that histamine injected intravenously exerted an anti-convulsant effect when used before Metrazol but had no effect upon electrically induced convulsion. It is interesting to note that anti-histamines apparently decrease the convulsive threshold, a fact which is becoming more generally recognized as patients who are on chlorpromazine therapy seem to develop spontaneous convulsions during insulin therapy more often than before.

These observations seem to infer that there are both quantitative and qualitative differences in the pre-convulsive cellular processes which follow the intravenous administration of Metrazol as opposed to those which follow electro-shock. Those differences might bring about selective clinical response in the two types of convulsive therapy and so justify the inclusion of *chemoplexy* in the therapeutic armamentatium of the general psychiatrist auxiliary to, and not necessarily replacing, electro-shock therapy. With the introduction of a technique which successfully eliminates the untoward discomfiture of previous methods this practice is commended to your most earnest attention.

The term *chemoplexy* requires consideration. Since venturing into private practice. I have become aware of a prejudice among the general public against the idea of shock-therapy, a term which was a misnomer from the start but which has stuck with the tenacity borne of constant usage. It has come to hold unpleasant implications and many patients who might well benefit from some type of convulsive therapy have had unfortunate associations with electro-shock treatments either directly or indirectly. They will often refuse to submit themselves to therapy because of their fear of electrical phenomena in general. Such patients agree to accept a form of treatment with the innocuous name of *chemoplexy* which guarantees freedom from any complicated electrical apparatus and which uses as a stimulant, not an electrical discharge, but a substance, as Metrazol, which has been effective in the treatment of respiratory and circulatory failure in surgical emergency and, among other things, is reported to have a revitalizing effect upon the general behavior of the aged.

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THE EFFECT OF SIMULATED ALTITUDE ON CERTAIN INSECT EGGS*

ARDZROONY PACKCHANIAN

The widespread and increasing use of aircraft represents a serious problem in the inadvertent transport of exogenous insects capable of transmitting diseases or of causing agricultural damage. The collection of live insects from the surface of aircraft has been reported by several investigators: Carneiro de Mendonça (1947), Griffitts (1931), Hughes (1949), Jarcho (1943), and Laird (1951, 1952). Dunnahoo (1944) and Laird (1950) have described the finding of insect egg masses transported on the outer surface of aircraft. Porter (1950) reported finding egg masses on 7.8 percent of over 9,000 aircraft examined in a nine-month period during 1949; the number of egg masses found per aircraft ranged from one to approximately one thousand.

Duval and Porter (1944) found that caterpillars of *Cossus cossus* could not survive temperatures below —21° C. Pictet (1893) reported that the eggs of *Bombyx mori* can be stored at —40° C. without harmful effect, while ant eggs were reported by the same author to have been killed by storage at 0° C.

Packchanian (1953, 1954) demonstrated that simulated altitude conditions of 10,000 feet (—5° C. with 522 mm. Hg) or below were without any marked detrimental effect on five species of insects; however, simulated altitude conditions at 30,000 feet (—44° C. with 225 mm. Hg) invariably killed all insects after 15 minutes exposure. It was concluded that if aircraft could fly at an altitude where the temperature of the surface could be kept at —40° C. or colder (due consideration being given to temperature increase caused by air friction) for half an hour, all insects on the surface would be killed.

The object of the present communication is to describe the

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effect of various simulated altitude conditions on the viability of eight species of insect eggs or pupae.

Materials and Methods

1. *High Altitude Chamber.* The equipment used for simulating various altitudes and the method of operation of this equipment have been described in detail in a previous communication (Packchanian, 1953).

2. *Insect Eggs.* The eggs of the following species of insects were used in these studies: (a) *Aedes aegypti*, the mosquito vector of yellow fever; (b) *Anopheles quadrimaculatus*, the mosquito vector of human malaria; (c) *Culex quinquefasciatus*, the mosquito vector of filariasis; (d) *Oncopeltus fasciatus*, the milkweed bug; (e) *Pediculus humanus corporis*, the human body louse and vector of typhus, plague, relapsing fever, etc.; and (f) *Rhodnius prolixus*, the blood sucker and potential vector of Chagas' disease.

3. *Insect Pupae.* The pupae of the following two species of insects were used in these studies: (a) *Musca domestica*, the common house fly, and (b) *Xenopsylla cheopis*, the Oriental flea (vector of typhus, plague, etc.).

4. *Rearing of Insects.* All of the above insects, with the exception of *Pediculus*, were reared in the laboratory from eggs to adults for at least several generations. The methods used differed somewhat from one species to another.

R. prolixus have been maintained in this laboratory for several years by feeding them on healthy rabbits. *Oncopeltus* were collected from oleander shrubs in Galveston, Texas, during 1953, and were reared in captivity for several generations by feeding them on milkweed seed and water.

X. cheopis are reared on rats, rat feces and dried, powdered blood. A rat (collared to prevent his eating the fleas) was placed in a wire cage which in turn was placed in a specially constructed cage with glass sides and a top of fine (150 mesh) wire gauze. The metal floor of the glass cage was kept covered with about two inches of sterile sand, the surface of which was covered with powdered blood and powdered rat feces, used as food for larvae. In this sand and fecal material, larvae and pupae of *Xenopsylla* flourished and fleas were at times so abundant that they fatally desanguinated the rat.

In general, the method of rearing the above species parallels standard procedures described in detail in entomological literature (Peterson, 1953).

5. *Collection and Exposure of Eggs or Pupae.* a. *Mosquitoes.* The three species of mosquitoes were reared in separate containers and were allowed to lay their eggs on moist filter paper. For exposure in the altitude chamber, small sections covered with mosquito eggs were cut from these filter papers and folded into small plastic cups about 3 cm. in diameter. After exposure at the required temperature and reduced pressure for a fixed period of time, the eggs were removed to large open petri dishes containing distilled water. The temperature in the hatching room was maintained at 25° C., and humidity was controlled at 80 to 88 per cent. Observations of the eggs were made for two weeks after exposure.

b. *Pediculus humanus corporis.* Eggs were obtained attached to small pieces of woolen cloth, sections of which were cut out and exposed in petri

plates containing a disc of filter paper. Immediately upon removal from the altitude chamber, the egg-covered wool was removed to a warmer petri plate containing some moistened cotton, and the dish was then placed in an incubator at 37° C. The eggs were observed daily after exposure for about two weeks and careful records were kept of the number of eggs hatching.

c. *Rhodnius prolixus.* Eggs of this reduviid bug were collected from the bottom of rearing jars and placed in small open pill boxes for exposure. After exposure, the eggs were removed from the pill boxes into test tubes, the bottom of which previously had been reinforced with cotton wrapped in gauze to prevent the eggs from coming into contact with the glass bottom. On top of this reinforced pad a few small strips of moist filter paper were placed to facilitate the movement of newly hatched nymphs. These test tubes were plugged with cotton and kept at room temperature (25° C.) for 20 to 40 days, observations for hatching being made at various intervals.

d. *Oncopeltus fasciatus.* Masses of milkweed bug eggs were collected from cotton nesting placed at the bottom of the rearing jars. Exposure was in small open pill boxes; hatching followed in test tubes as described above for *Rhodnius.*

e. *Xenopsylla cheopis* pupae were collected from the rearing cage by vacuum siphoning into a flask. The pupae were then transferred to small open pill boxes for exposure in the altitude chamber. After exposure, pupae were transferred from the pill boxes to warm cotton-plugged test tubes which were then maintained at 25° C. for about two weeks, during which time daily observations were made for emergence of fleas.

f. *Musca domestica* pupae were collected from the rearing cage and exposed and observed as described above for fleas.

Special effort was made to collect eggs freshly laid from fertile parents. Each batch was divided into several groups, some of which were used for exposure in the altitude chamber under various conditions while others were kept as controls under conditions as described above for hatching of the various species.

Whenever possible, hundreds of eggs were used for each simulated altitude test. In some species, smaller numbers were used and hatching was determined by counting; where more than 50 were used in one batch, the percentage hatching was estimated.

Experimental Data. The results of these studies are summarized in tables 1 through 8. A careful study of these data reveals the following facts pertaining to insect eggs and pupae.

1. The Effects of Various Simulated Altitudes on Insect Eggs.

a. *Effect of Simulated Altitude on* Aedes aegypti *Eggs.* (See Table 1.) The eggs of this species, exposed to conditions simulating an altitude of 12,500 feet (3,810 meters) above sea level (—10° C. with absolute pressure of 472 mm. Hg), survived one hour but failed to hatch after two hours of exposure. Fifteen min-

utes of exposure to a temperature of —15° C. with absolute pressure of 429 mm. Hg failed to kill all of the eggs exposed, but exposures of 30 minutes to one hour at these conditions resulted in the failure of all exposed eggs to hatch.

Other batches of eggs were all killed when exposed for 15 minutes at —20° C. with 390 mm. Hg, to —25° C., and —30° C., each with corresponding reduced pressures.

TABLE 1

The Effects of Simulated Altitude Conditions on Aedes aegypti *Eggs*

Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Total Exposure Min.	No. Eggs Exposed (Approx.)	Eggs Hatched
—10	472	88	.	15	20	+
—10	472	88	.	30	20	+
—10	472	88	1	..	20	+
—10	472	88	2	..	20	0
—15	429	83	.	15	20	+
—15	429	83	.	30	20	0
—15	429	83	1	..	20	0
—20	390	72	.	15	20	0
—20	390	72	.	30	20	0
—25	349	63	.	15	20	0
—25	349	63	.	30	20	0
—30	310	63	.	15	20	0
—30	310	63	.	30	20	0
—44	225	62	.	15	20	0
—44	225	52	.	30	20	0
+25	760	88	Unexposed Controls		20	+

At —44° C., with an absolute pressure of 225 mm. Hg, corresponding to 30,000 feet altitude or 9,135 meters, 15 minutes of exposure invariably killed all eggs exposed.

Unexposed control eggs, kept at room temperature in a suitable environment, all hatched.

b. *Effect of Simulated Altitude on* Anopheles quadrimaculatus *Eggs.* (See Fig. I-A and Table 2.) Batches of eggs, exposed to —15° C. with an absolute pressure of 429 mm. Hg for 15 minutes, 30 minutes and one hour, were not all killed. However, after two hours under the same conditions, all eggs were dead.

When the temperature was reduced to —20° C. with 390 mm.

Hg absolute pressure, 15 to 30 minutes exposure likewise was not completely effective; however, one to two hours of exposure prevented any of the eggs from hatching.

At —25° C. with 349 mm. Hg, 15 minutes of exposure failed to prevent all the eggs from hatching. When the period was extended to 30 minutes or longer, all eggs failed to hatch.

At —30° C. and —44° C. with corresponding reduced pressure, 15 minutes of exposure was sufficient to kill all eggs exposed.

TABLE 2

The Effects of Simulated Altitude Conditions on Anopheles quadrimaculatus *Eggs*

Conditions in Altitude Chamber					Results	
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Eggs Exposed (Approx.)	Eggs Hatched Percent
—15	429	81	.	15	100	80
—15	429	75	.	30	100	60
—15	429	75	1	..	100	10
—15	429	75	2	..	100	0
—20	390	71	.	15	100	50
—20	390	71	.	30	100	10
—20	390	71	1	..	100	0
—20	390	71	2	..	100	0
—25	349	68	.	15	100	10
—25	349	68	.	30	100	0
—25	349	68	1	..	100	0
—25	349	68	2	..	100	0
—30	310	54	.	15	100	0
—30	310	54	.	30	100	0
—44	225	49	.	15	100	0
—44	225	49	.	30	100	0
+25	760	88	Unexposed Controls		100	80

Unexposed controls hatched when kept under ideal conditions at room temperature.

c. *Effect of Simulated Altitude on* Culex quinquefasciatus *Eggs.* (See Table 3.) Some eggs of this species survived exposures varying in length from 15 minutes to two hours at a temperature of —10° C., with absolute pressure at 472 mm. Hg. Not all eggs were killed when exposed to —15° C. with 429 mm. Hg, for 30 minutes, but one hour under these conditions killed all the eggs exposed.

Culex eggs exposed for 15 minutes at —20° C. with 387 mm. Hg were hatched, but 30 minutes was detrimental to the eggs at these simulated altitude conditions.

No eggs of this species survived exposures of 15 minutes at —25° C. or at —30° C., with corresponding reduced pressures. Likewise, eggs of *Culex* exposed for 15 minutes at conditions simulating an altitude of 30,000 feet (—44° C. with 225 mm. Hg) all failed to hatch.

Uneventful hatching of unexposed controls was obtained.

d. *Effect of Simulated Altitude on* Oncopeltus fasciatus *Eggs.* (See Table 4.) The majority of the eggs of this species hatched after exposures of two to four hours at —5° C. with 522 mm. Hg absolute pressure. At —10° C. with 472 mm. Hg, after exposure periods of 15 minutes, 30 minutes, or one hour, 10 to 70 percent of the eggs hatched, but after two hours of pressure at this same simulated altitude (12,500 feet) all eggs were killed.

About 20 percent of *Oncopeltus* eggs survived exposure for 30 minutes at —15° C. and 429 mm. Hg absolute pressure (conditions simulating 15,000 feet altitude) but no survivors were noted after exposures of one hour or longer at these same conditions.

At —20° C. with 387 mm. Hg or at —25° C. with 349 mm. Hg, 15 minutes of exposure or longer likewise prevented the eggs from hatching.

Unexposed controls hatched when kept at room temperature.

e. *Effect of Simulated Altitude on* Pediculus humanus corporis *Eggs.* (See Fig. I-b and Table 5.) Some eggs of this species survived exposures of 15 minutes to two hours at a simulated altitude of 15,000 feet (—15° C. with 429 mm. Hg absolute pressure). At —20° C. with 387 mm. Hg survivals were noted after exposures of 15 and of 30 minutes, but no eggs hatched after one or two hours of exposure at the latter conditions.

At —25° C. with 349 mm. Hg absolute pressure, 30 minutes of exposure failed to kill all eggs tested. None of the eggs hatched when exposed to this same environment for one hour or longer.

Test conditions in the altitude chamber of —30° C. with 310 mm. Hg and of —44° C. with 225 mm. Hg killed all eggs exposed for 15 minutes.

A large percentage of unexposed controls hatched.

f. *Effect of Simulated Altitude on* Rhodnius prolixus *Eggs.* (See Fig. I-c and Table 6.) At conditions simulating 10,000 feet altitude (—5° C., 522 mm. Hg), 100 perecent of eggs hatched after 15 minutes of exposure. When the exposure period was increased from 15 minutes to two hours, the percentage hatching dropped from 100 to 20 percent. Four hours or longer at the same simulated altitude conditions invariably fatally harmed the eggs and none hatched.

TABLE 3

The Effects of Simulated Altitude Conditions on Culex quinquefasciatus *Eggs*

Conditions in Altitude Chamber					Results	
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Eggs Exposed (Approx.)	Eggs Hatched
—10	472	88	.	15	100	+
—10	472	88	.	30	100	+
—10	472	88	1	..	100	+
—10	472	88	2	..	100	+
—15	429	83	.	15	100	+
—15	429	83	.	30	100	+
—15	429	83	1	..	100	0
—15	429	86	2	..	100	0
—20	390	72	.	15	100	+
—20	390	72	.	30	100	0
—25	349	64	.	15	100	0
—25	349	64	.	30	100	0
—30	310	54	.	15	100	0
—30	310	54	.	30	100	0
—44	225	56	.	15	100	0
—44	225	56	.	30	100	0
+25	760	88	Unexposed Controls		100	+

At —10° C. with 472 mm. Hg pressure, 30 percent of the eggs tested were hatched after 15 and 30 minutes exposure, 20 percent after one hour and none after two hours. In experiments conducted at —15° C. with 429 mm. Hg, exposures of 15 minutes killed 60 percent of the eggs; while 30 minutes at the same altitude conditions killed all of them.

Simulated altitudes of 17,500 feet (—20° C. with 387 mm. Hg), 20,000 feet (—25° C. with 349 mm. Hg), and 22,500 feet

(—30° C. with 314 mm. Hg) above sea level were fatal to all *Rhodnius* eggs; none hatched after exposure periods of 15 minutes or longer.

Tests made at —44° C. with 225 mm. Hg likewise killed all eggs exposed for 15 minutes.

About 85 percent of several control lots of *Rhodnius prolixus* eggs kept at room temperature (25° C.) hatched.

TABLE 4

The Effects of Simulated Altitude Conditions on Oncopeltus fasciatus *Eggs*

Conditions in Altitude Chamber					Results	
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Eggs Exposed (Approx.)	Eggs Hatched Percent
—5	522	90	2	..	11	100
—5	522	88	4	..	12	83
—10	472	79	.	15	10	70
—10	472	79	.	30	10	50
—10	472	75	1	..	10	10
—10	472	75	2	..	10	0
—15	429	73	.	15	10	50
—15	429	75	.	30	5	20
—15	429	75	1	..	6	0
—15	429	75	2	..	7	0
—20	390	60	.	15	10	0
—20	390	71	.	30	7	0
—20	390	71	1	..	7	0
—20	390	71	2	..	6	0
—25	349	69	.	15	12	0
—25	349	68	.	30	6	0
—25	349	68	1	..	5	0
—25	349	68	2	..	5	0
+25	760	75	Unexposed Controls		110	89

2. *The Effects of Various Simulated Altitudes on Insect Pupae. a. Effect of Simulated Altitude on* Musca domestica *Pupae.* (See Table 7.) Eighty-five percent of *Musca domestica* pupae survived and emerged after a four-hour exposure at a temperature of —5° C. with 522 mm. Hg absolute pressure.

At —10° C., with 472 mm. Hg absolute pressure, after 15 minutes in the altitude chamber, 67 percent of pupae exposed were killed; after 30 minutes, all were killed.

At —15° C., with 429 mm. Hg absolute pressure (15,000 feet

equivalent altitude) 11 percent of pupae were able to emerge after an exposure of 15 minutes, but all were killed after 30 minutes or longer at these same conditions.

Exposures of 15 minutes or longer at a simulated altitude of 17,500 feet above sea level (—20° C. with 390 mm. Hg absolute pressure) killed all pupae tested.

A large percentage of unexposed controls kept at 25° C. emerged as adult flies.

TABLE 5

The Effects of Simulated Altitude Conditions on Pediculus humanus corporis *Eggs*

Conditions in Altitude Chamber					Results
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr. Min.	No. Eggs Exposed (Approx.)	Eggs Hatched
—15	429	81	. 15	20	+
—15	429	82	. 30	20	+
—15	429	75	1 ..	20	+
—15	429	80	2 ..	20	+
—20	390	71	. 15	100	+
—20	390	71	. 30	100	+
—20	390	71	1 ..	100	0
—20	390	71	2 ..	20	0
—25	349	68	. 30	100	+
—25	349	68	1 ..	100	0
—25	349	68	2 ..	20	0
—30	310	53	. 15	100	0
—30	310	53	. 30	100	0
—44	225	49	. 15	100	0
—44	225	49	. 30	100	0
+25	760	75	Unexposed Controls	100	+

b. *Effect of Simulated Altitude on* Xenopsylla cheopis *Pupae.* (See Fig. I-E and Table 8.) Pupae of this species survived and emerged after exposures of from 15 minutes to two hours, at a temperature of —10° C., with absolute pressure at 472 mm. Hg. Some pupae survived after 15 minutes in the altitude chamber at a simulated height of 15,000 feet (—15° C. with 429 mm. Hg), but longer exposures at this simulated altitude killed all pupae tested.

No viable pupae were noted after tests at —20° C., —25° C.,

or −30° C., all with corresponding reduced pressures, in tests of from 15 minutes to two hours in duration. Likewise, a simulated altitude of 30,000 feet above sea level (−44° C. with 225 mm. Hg absolute pressure) killed all pupae tested for 15 minutes or longer.

Unexposed controls developed and emerged normally.

Discussion. Careful analysis of the data of this study reveal minor variations in resistance to exposure to simulated altitude conditions among the eggs or pupae of the insect species studied.

TABLE 6

The Effects of Simulated Altitude Conditions on Rhodnius prolixus *Eggs*

Conditions in Altitude Chamber						Results
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Eggs Exposed	Eggs Hatched
−5	522	92	.	15	9	100
−5	522	92	.	30	10	90
−5	522	92	1	..	10	80
−5	522	92	2	..	10	20
−5	522	92	4	..	10	0
−5	522	92	8	..	10	0
−5	522	92	24	..	10	0
−5	522	92	48	..	10	0
−10	472	88	.	15	10	30
−10	472	88	.	30	10	30
−10	472	88	1	..	10	20
−10	472	88	2	..	10	0
−15	429	80	.	15	10	40
−15	429	80	.	30	10	0
−15	429	80	1	..	10	0
−15	429	80	2	..	10	0
−20	390	70	.	15	10	0
−20	390	70	.	30	10	0
−20	390	70	1	..	10	0
−20	390	70	2	..	10	0
−25	349	68	.	15	10	0
−25	349	68	.	30	10	0
−25	349	68	1	..	10	0
−25	349	68	2	..	10	0
−30	310	63	.	15	10	0
−30	310	63	.	30	10	0
−44	225	52	.	15	10	0
−44	225	52	.	30	10	0
+25	760	75	Unexposed Controls		69	85

The most resistant appeared to be the eggs of *Pediculus humanus corporis*, some of which survived and hatched after 30 minutes of exposure at —25° C. with 349 mm. Hg absolute pressure (conditions simulating 20,000 feet above sea level). A limited number of experiments also revealed that the eggs of *Amblyomma americanum*, an arthropod (see Fig. I-D) tolerated a temperature of —30° C. with 310 mm. Hg absolute pressure (corresponding to simulated altitude of 22,500 feet) for 30 minutes. The majority

TABLE 7

The Effects of Simulated Altitude Conditions on Musca domestica *Pupae*

Conditions in Altitude Chamber					Results	
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Pupae Exposed	Pupae Hatched Percent
—5	522	85	.	..	13	85
—10	472	79	.	15	6	33
—10	472	79	.	30	6	0
—15	429	73	.	15	9	11
—15	429	75	.	30	3	0
—15	429	75	1	..	3	0
—15	429	75	2	..	3	0
—20	390	60	.	15	11	0
—20	390	71	.	30	3	0
—20	390	71	1	..	3	0
—20	390	71	2	..	3	0
+25	760	75	Unexposed Controls		30	77

of the eggs of other species tested tolerated a minimum temperature of —15° C. with pressure at 429 mm. Hg for periods of 15 to 30 minutes only. However, all of the eggs or pupae of the insect species studied were killed after exposures of 15 minutes at a simulated altitude of 30,000 feet (temperature —44° C. with 225 mm. Hg absolute pressure).

In previous studies in this laboratory of the effect of altitude conditions on insects (Packchanian, 1953), it was demonstrated that low temperature alone and not humidity or reduced pressure was the chief factor responsible for the death of insects. In all probability, the same factor, namely, low temperature, is alone responsible for the detrimental effect of simulated altitude con-

ditions on insect eggs. This assumption concurs with the findings of most early investigators; an admirable summary of the literature on this subject can be found in the reviews of Luyet and Gehenio (1938, 1940).

In the present studies, no corrections have been made for the heat generated by friction on the surface of aircraft during flight. This heat of friction may cause the temperature of the surface to rise many degrees above the atmospheric temperature at the level

TABLE 8

The Effects of Simulated Altitude Conditions on Xenopsylla cheopis *Pupae*

Conditions in Altitude Chamber					Results	
Temp. °C.	Absolute Pressure mm. Hg.	Corrected Rel. Hum. Percent	Total Exposure Hr.	Min.	No. Pupae Exposed	Pupae Hatched Percent
—10	472	88	.	15	8	13
—10	472	88	.	30	10	20
—10	472	88	1	..	10	20
—10	472	88	2	..	16	6
—15	429	76	.	15	12	17
—15	429	76	.	30	14	0
—15	429	76	1	..	9	0
—15	429	76	2	..	8	0
—20	390	67	.	15	12	0
—20	390	67	.	30	5	0
—20	390	67	1	..	1	0
—20	390	67	2	..	5	0
—25	349	63	.	15	11	0
—25	349	63	.	30	7	0
—25	349	63	1	..	21	0
—25	349	63	2	..	9	0
—30	310	63	.	15	20	0
—30	310	63	.	30	4	0
—44	225	52	.	15	15	0
—44	225	52	.	30	6	0
+25	760	75	Unexposed Controls		12	25

of flight. The magnitude of this temperature rise naturally varies with the altitude and with the surface and speed of the aircraft.

Since live insect eggs have been collected from commercial aircraft by a number of investigators (Laird, 1950, 1951, 1952; Porter and Hughes, 1950), one must assume, in the light of the present studies, that such aircraft conducted their flights at lower

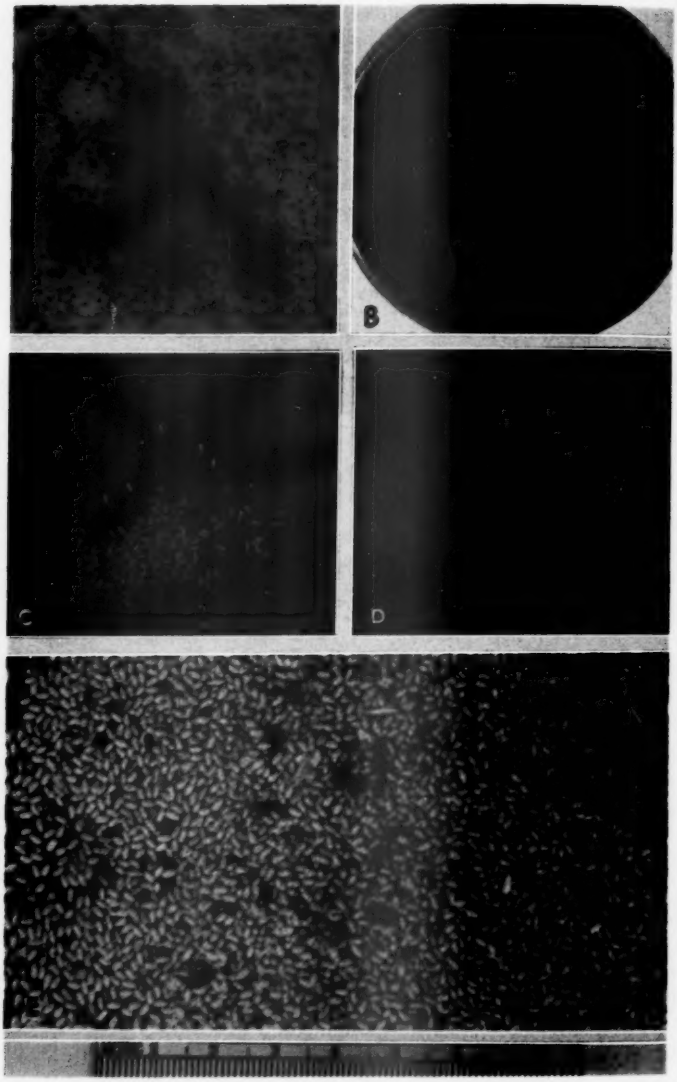

FIG. I. Arthropod Eggs and Pupae (photographed natural size): (a) *Anopheles quadrimaculatus* (eggs); (b) *Pediculus humanus corporis* (eggs); (c) *Rhodnius prolixus* (eggs); (d) *Amblyomma americanum* (eggs); and (e) *Xenopsylla cheopis* (pupae).

altitudes, probably below 15,000 feet, where the surface temperature of the aircraft in all probability was not below —15° C. Another factor that may have contributed to survival of insect eggs on aircraft surfaces at low altitudes is possibly the heat generated by friction which may raise the temperature of the surface above the lethal level, as discussed above.

The experimental data on hand and earlier publications of the writer suggest that in certain conditions, when the pilot is aware that his aircraft is infested with insects on departure, particularly during war time, one of the effective methods of preventing the transportation of undesirable exogenous insects and their eggs lodged on the surface of aircraft would be to maintain an altitude of about 30,000 feet above sea level (where the temperature is normally —44° C. and atmospheric pressure is 225 mm. Hg) for 15 to 30 minutes. This altitude should be reached immediately after the aircraft leaves an insect infested area before crossing into new and uninfested regions. This method of killing insects on the surface of aircraft during flight is an added precaution and will not replace the standard procedures of aircraft disinsectization and disinfection on arrival of the aircraft at airports.

Summary

1. Eggs or pupae of the following eight species of insects were exposed to various simulated altitude conditions in an altitude chamber (refrigerated vacuum chamber), and the effects of such conditions on the viability of the eggs or pupae were determined: *Aedes aegypti, Anopheles quadrimaculatus, Culex quinquefasciatus, Oncopeltus fasciatus, Pediculus humanus corporis, Rhodnius prolixus* and pupae of *Musca domestica* and *Xenopsylla cheopis.*

2. Injurious effects of certain altitudes to eggs or pupae differed somewhat in different species. Some survived several hours of exposure to conditions simulating 15,000 feet above sea level (—15° C. with 429 mm. Hg absolute pressure), while the same conditions were apparently lethal to other species.

3. Exposure to —30° C. with 314 mm. Hg absolute pressure (22,500 feet above sea level) or to —44° C. with 225 mm. Hg absolute pressure (30,000 feet above sea level) for 15 minutes, was

detrimental to all eggs or pupae of the species studied; none hatched after such exposures.

4. Altitude conditions below 22,500 feet (—30° C. with absolute pressure of 310 mm. Hg) required longer exposure periods to kill insect eggs or pupae of the species studied.

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STUDY BY ELECTRON MICROSCOPY OF ERYTHROCYTES FROM CAVE BATS *(MYOTUS LUCIFUGUS)* INFECTED INTRAPERITONEALLY WITH YELLOW FEVER VIRUS (17 D STRAIN)*

REGINALD L. REAGAN, SING CHANG AND A. L. BRUECKNER

The 17 D strain of yellow fever virus used for this study was furnished by the American Type Culture Collection of Washington, D. C. This virus had originally been obtained from Dr. Sawyer of the Rockefeller Foundation Laboratories. The 229th passage of the 17 D strain was received at the Rocky Mountain Laboratory from the Rockefeller Foundation as Lot 145–3AB769. The strain has been through 5 passages in embryonated eggs at the Rocky Mountain Laboratory, Hamilton, Montana. The 17 D strain is widely used for immunization of man against yellow fever. It produces no known visceral lesions in man or monkey when inoculated subcutaneously, but induces a mild systemic disease with small amounts of circulating virus (Theiler, 1930).

Materials and Methods

The virus was received here in lyophilized chick embryo tissue and was diluted to a 10 per cent suspension with physiologic saline solution.

The experiment was conducted to see if virus could be seen on or in the erythrocytes of cave bats exposed to this strain, and it was conducted in the following manner. Ten healthy cave bats *(Myotus lucifugus)*, age unknown, were divided into two groups. They were designated as Group 1 and Group 2. One-tenth cc. of the previously described inoculum was injected intraperitoneally into each bat of Group 1. One-tenth cc. of normal chick embryo tissue was injected into bats of Group 2, control group. The cave bats from Group 1 were bled from the heart twenty-four hours after exposure to the virus and the blood was pooled and placed in 2 cc. of heparinized saline solution. The cave bats (control) were treated in the same manner. Table 1 shows the interval bleedings. Each pooled blood specimen from Group 1 and Group 2, from all interval bleedings, was prepared for electron microscopic examination by placing small drops of the erythrocytic suspension in distilled water for twenty seconds, then placing this material on parlodion film

* From the Virus Laboratory, University of Maryland, College Park, Maryland. Received for publication April 18, 1955.

supports which had been prepared two weeks previously. The films were dried and shadowed with chromium (Williams, *et al.*, 1946) at arc tangent 2/12 and examined under an RCA electron microscope, type EMU. The same procedure described above was carried out at 48, 72, 96, 120, 144, 168, and 192 hours for test and control groups. The bats showed no symptoms of yellow fever disease during the entire period, and were discarded after a fifteen-day observation period. The bloods from all interval bleedings were injected intracerebrally into Swiss albino mice (3 weeks of age). Mice show central nervous disease symptoms when inoculated intracerebrally with the original 17 D strain of yellow fever virus. The experimental results of the mice inoculation are given in table 1.

TABLE 1

Interval Bleedings from Test and Control Group Cave Bats and Response of Swiss Albino Mice Injected Intracerebrally with the Test Group Interval Bleeding Stated Below

Bleeding Interval	Group 1 Virus Present in or on Erythrocyte	Group 1 Mice Injected Intracerebrally No. Injected	Group 1 Mice Injected Intracerebrally No. Showing Symptoms	Group 2 Virus Present in or on Erythrocyte
24 hours....	Pos.	6	6	0
48 hours....	Pos.	6	6	0
72 hours....	0	6	0	0
96 hours....	0	6	0	0
120 hours....	0	6	0	0
144 hours....	0	6	0	0
168 hours....	0	6	0	0
192 hours....	0	6	0	0

Results and Discussion

Upon examination of the erythrocytes under the electron microscope, no virus-like particles were observed for the control Group 2 bats (at hourly intervals stated) which had received normal chick embryo tissue intraperitoneally. A representative erythrocyte from one of the control bleedings is given in figure I. Virus-like particles were seen on or in the erythrocyte suspension of the test Group 1 at 24 hours and 48 hours, as shown in figure II and figure III. No virus-like particles were seen on or in the erythrocyte of the other interval bleedings. The blood suspension for the 24 and 48 hour bleedings after injection intracerebrally in Swiss mice produced an encephalitis in these animals. This is usually found when the original 17 D strain of yellow fever virus is injected intracerebrally in Swiss albino mice.

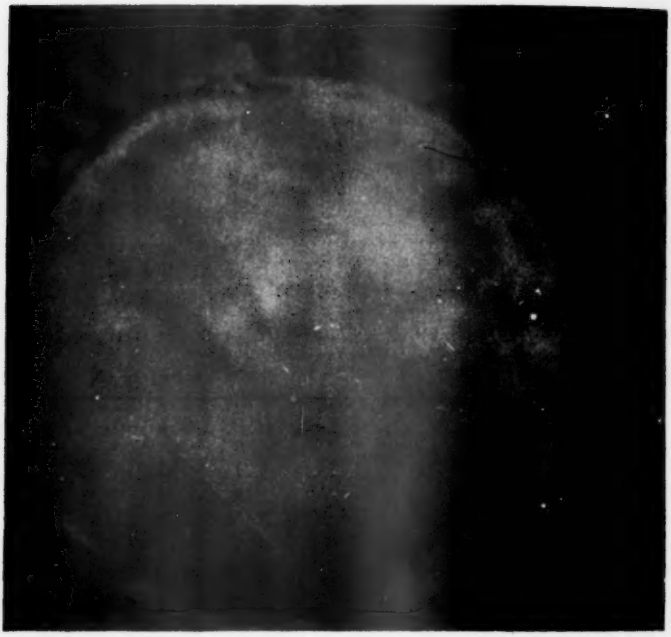

FIG. I. Electron micrograph from one of the control bleedings (48 hour) showing no virus-like particles on or in the erythrocyte. Shadowed with chromium at arc tangent 2/12 x 30,000.

Summary. In a previous paper (Reagan, *et al.*, 1953), we described this purified 17 D yellow fever strain to be round with a slightly irregular contour and with a diameter of 50 to 55 mu and to resemble the virus of measles (morbilli) described by Reagan, *et al.*, 1952. The virus in or on the erythrocyte in this experiment from the 24 and 48 hour bleeding intervals show the virus particles to resemble the original purified 17 D virus described above. The 24 and 48 hour bleedings produced an encephalitis in Swiss mice injected intracerebrally. The other bleedings from the control and infected group showed no virus-like particles on or in the erythrocyte, and their suspensions were not infective for Swiss mice inoculated intracerebrally.

FIG. II. Electron micrograph from the 24-hour test group showing virus-like particles on or in the erythrocyte, indicated by arrows. Shadowed with chromium at arc tangent 2/12 x 30,000.

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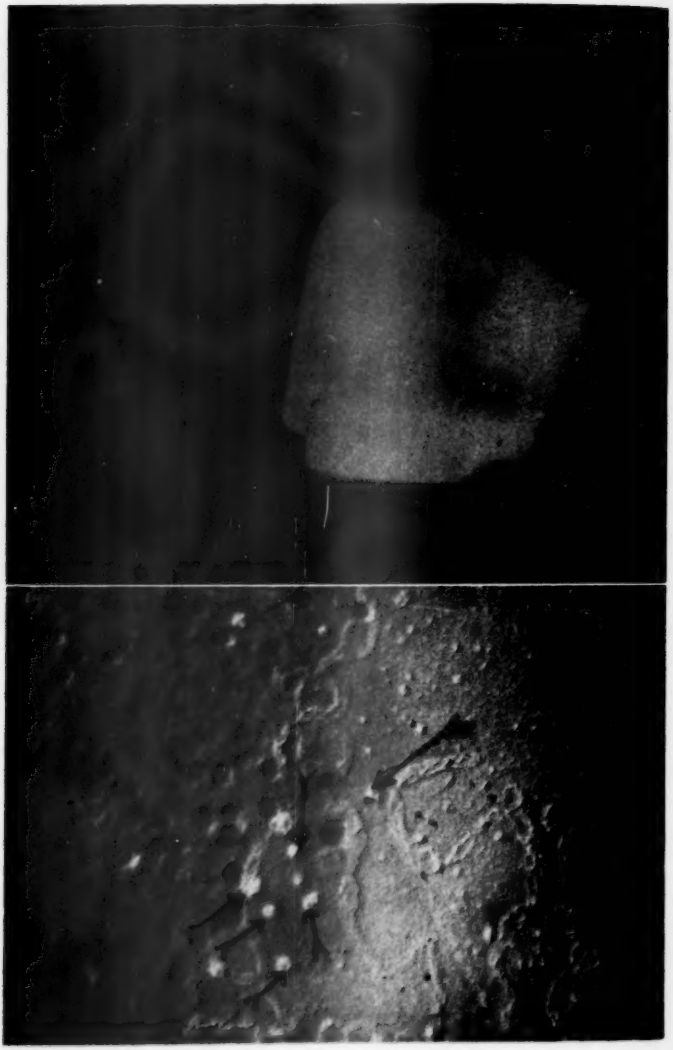

FIG. III. Electron micrograph from the 48-hour test group showing virus-like particles on or in the erythrocyte, indicated by arrows. Shadowed with chromium at arc tangent 2/12 x 30,000.

A *VARIANT PINOCYTIC CELL (VP CELL)* OF GEY'S STRAIN HELA SELECTIVELY PRODUCED AND STIMULATED BY HUMAN SERUM NUTRIENTS*

GEORGE G. ROSE

This report introduces a cytological response which was first observed through the phase contrast microscope while viewing cultures of Gey's strain HeLa (1952 and 1954). The cells were routinely cultivated in a benign ascitic fluid nutrient and as such did not demonstrate the particular cell entity to be described. The production of this cell which has been termed a *variant pinocytic cell (VP cell)* was apparently a direct effect of human serum nutrients.

The assumption that *factor X* is present in sera from cancerous patients and absent or minimally present in sera from non-cancerous individuals has intrigued many investigators. An inestimable number of man-hours of vigilant research with a resultant mass of reports has followed this trend of thought. Although this unknown has yet to be found, pursuit of its presence is still desirable in this era of threshold cancer knowledge. Wynne, Gott, and Russell (1954) attacked this assumption through a microbiological approach in which bacterial strains were subjected to serum tungstic acid filtrates. Hill and Levi (1954) formulated a procedure for assaying a catalytic serum component which they found to be low in normal or non-cancerous hosts and high in malignancies and pregnancies. The serological reaction was reemphasized by Eisenstaedt (1954) and by Gorer and Mikulska (1954).

Because the laborious efforts preceding this report have not definitely identified an exclusive cancer agent from the blood serum, it would appear that should *factor X* be real its particular

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value must be extremely complex and as such would demand an unusual and delicate method of detection. This author has held for some time that a necessary approach toward finding this detection method was to investigate physiological responses as recorded through the medium of tissue culture. What could render a more sensitive and delicate response than the functional unit of life itself, the human cell? As the sensitive element the cell responds to the most minute environmental change yet always recovers to its stable form or succumbs to this change. Rarely, a cell changes character as a result of the environmental shift without returning to its stable form. These character changes may be considered as failures of biological thrusts toward evolutionary perfection, degradation products which hold populations in an environmental balance and the fulminant seeds of the malady we all recognize too well.

The idea of exposing tissue cultures to sera is not new. Carrel (1934) showed that buffy-coat cultures assumed a characteristic growth pattern dependent upon the serum in which they were being cultivated rather than upon the origin of the cells. Tumor tissue cultures were inhibited by cancerous sera as reported by Woodhouse (1940) and twelve years later Sacerdote (1952) showed that malignant sera stimulated mitosis of normal tissues *in vitro*. Ulloa-Gregori, *et al.* (1950), demonstrated the growth promotion qualities of malignant ascitic fluid as compared to that of benign origin in tissue cultures of human skin. Recently, Cailleau and Kirk (1954) reported contrary observations in cultures of Earle's strain L cells.

In attempting to determine a differential response of cells to malignant sera versus non-malignant sera a very close scrutiny of the cells under attack was made with phase contrast microscopy. Using multipurpose culture chambers (Rose, 1954) which allowed phase contrast visualization throughout the cultivation period, Gey's strain HeLa was observed under the aegis of these two types of sera as well as under the influence of basic nutrients composed of benign ascitic and pleural fluids.

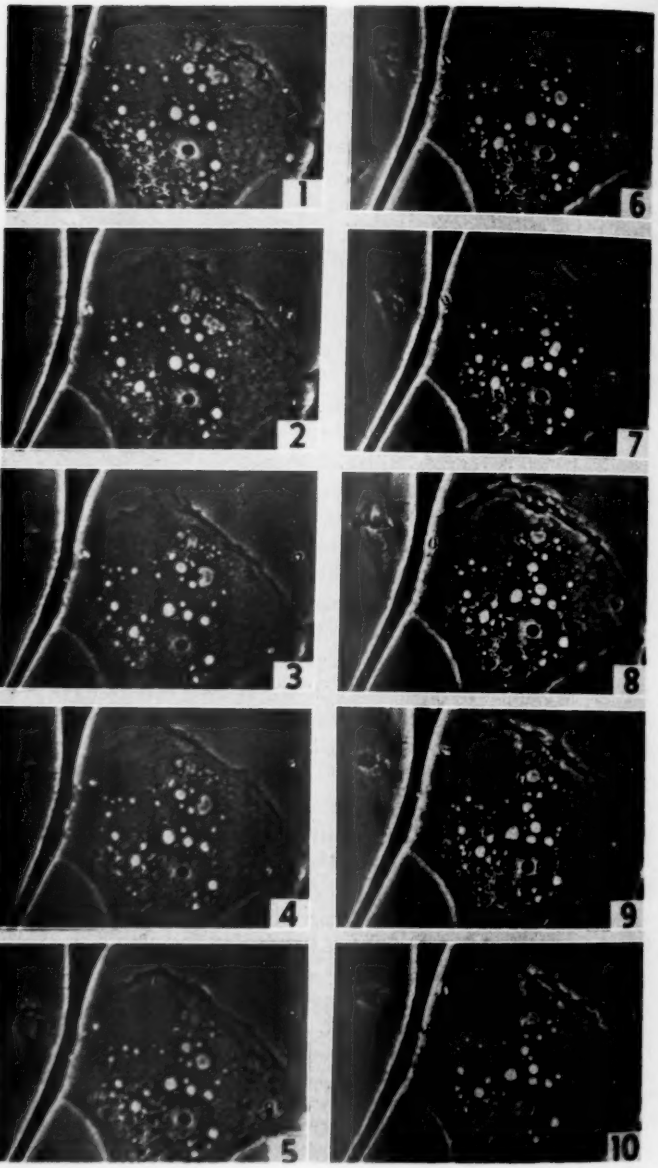
Pinocytosis was first described by Lewis (1931) and with the subsequent development of phase contrast optical systems, it has been studied extensively by Pomerat (1953) in cinematographic recordings. This cell drinking or *pinocytosis* was described by

Gey (1954) as an ingestion of droplets from the fluid medium which passed beneath the limiting plasma cell membrane and moved directly in the hyaloplasm streaming to the juxtanuclear *(Golgi)* zone. Three features were noted in this transit and they were (1) a change from an irregular mass to a round form, (2) a rapid reduction in size and (3) a gradual shift in their refractive index. Figures 1–10 and 11–20 adequately corroborate Gey's first two features. The refractive index shift reversed the phase from white to black or vice versa depending upon the phase system used. Gey also observed a fusion of these droplets with mitochondria as they diminished in size.

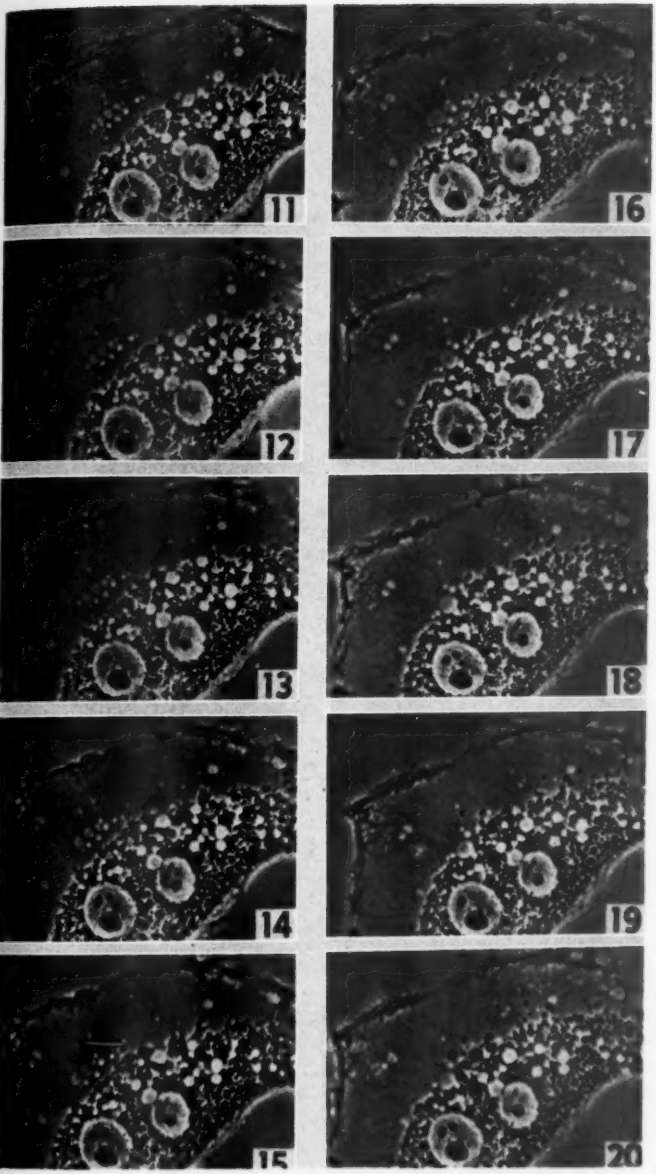
The production of the *variant pinocytic cell (VP cell)* by serum nutrients was apparently some alteration of Gey's second feature, viz., a reduction in size. Although the fluid droplets were frequently seen to coalesce (Figs. 13–16) before the shift in the refractive index, this coalition was not generally thought to occur afterwards with a subsequent enlargement phenomenon of the juxtanuclear granules. Some cells contained very few enlarged granules (Fig. 22) which demonstrated that their production was not due to a coalescence in closely packed areas. Time-lapse cinematographic recordings of the *VP cell* production have not been made at this time but enough visual observations have been made to strongly suggest that this cell was germinated as a result of fluid absorption with the ultimate swelling of these intracellular granules. Usually these cells were undergoing an excessive *pinocytosis* and as the granules accumulated the cell drinking became even more pronounced until the cell was literally stuffed. Here the index of refraction apparently was realtered so that the granules brightened (B&L optics) under phase contrast illumination (Figs. 23–26). At this point the cell became a dying system, a granular bag which soon fell away with other debris.

Materials and Methods

Stock HeLa cultures were maintained in roller tubes with 2.5 cc. of a nutrient composed of Earle's balanced salt solution (50 per cent) and benign ascitic or pleural fluid (50 per cent). These nutrients also contained penicillin G (1,000 u/cc.) as an asceptic precaution and phenol red (0.001 per cent) as the pH indicator. When the stock colonies reached a diameter of 5 mm. or greater they were used for seeding the chambers. The stock colonies were scraped from the walls of the tubes and then poured into a Petri dish. After cutting these net-like cultures into fragments approximately 1 mm. in diam-



FIGS. 1 to 10. A typical cell of strain HeLa undergoing pinocytosis in a benign ascitic fluid nutrient. Photographs were taken at one-minute intervals so that the fluid droplets may be traced with ease as they move toward the juxtanuclear zone. Note the minute size of the dark juxtanuclear granules.



FIGS. 11 to 20. A typical *variant pinocyte (VP cell)* of strain HeLa as recorded in a malignant serum nutrient. Photographs were taken at one-minute intervals. Compare the large size of the juxtanuclear granules in this cell with those in the cell illustrated in figures 1 to 10.

FIGS. 13 to 16. Note the coalescing of the cytoplasmic fluid droplets before they enter the juxtanuclear zone.

eter, groups of four multipurpose culture chambers were planted with the use of a rubber-bulbed pipette. Each chamber received one central fragment and after closing the chambers they were filled with approximately 1.8–2.0 cc. of the basic nutrient described above. After this the cultures were incubated at 34–38° C. for two or three days when they were inverted and remained in this hanging position throughout the experimentation period. This inversion allowed the debris of metabolism and degeneration to fall away from the cultures which assisted in eliminating mechanical barriers of growth as well as in improving optical visualization. Ideally, a 1 mm. sized fragment would pyramid into a circular colony 4–5 mm. in diameter within seven to ten days. Occasionally the culture took ten to thirteen days to assume this proportion. Probably the major difference in time was due to a difference in the number of viable explanting cells. Rarely a colony regressed after a seemingly successful start and eventually succumbed in an alkalinized chamber. Rapidly growing colonies discharged carbon dioxide into the fluid medium at a greater rate than the alkaline absorption from the glass and the buffer action were able to stabilize. This resulted in a steady acid shift which in some cases required a corrective fluid exchange. The initiating nutrient was usually at pH 7.8–8.1 when added to the chambers and in five to seven days had fallen to pH 7.5–7.7.

Essentially replicate cultures were selected for the serum analyses as determined by (1) their cross-sectional diameters and (2) the degree of pH shift toward acid. This selection was made between the seventh and thirteenth day following chamber explantation. Chambers selected were evacuated of their nutrients using a 2 cc. syringe fitted with a No. 25 needle. The syringe needle and another No. 25 needle were inserted through the edge of the rubber gasket and after nutrient removal the fresh nutrient was supplied using another syringe and No. 25 needle. The unattached needle

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FIG. 21. Photograph of a stage micrometer which may be used as a reference for all of the illustrations (Figs. 1 through 31). Each division represents 0.01 mm.

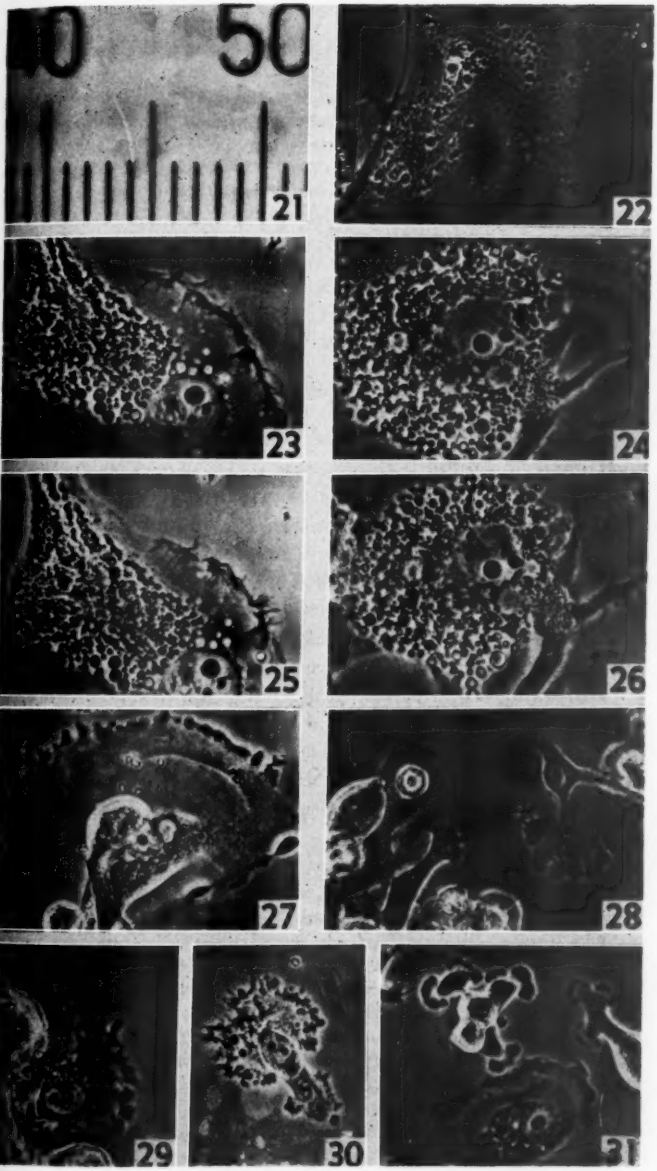
FIG. 22. *VP cell* in an early form. This demonstrates that the large juxtanuclear granules of this cell type were not produced by a fusion of lesser granules in a closely packed area.

FIGS. 23 and 25. *VP cell* in a late form. Photographs were taken at ten-minute intervals.

FIGS. 24 and 26. *VP cell* in a late form. Photographs were taken at ten-minute intervals.

FIGS. 27 to 31. Degenerative forms of strain HeLa after three days in the presence of a benign ascitic fluid nutrient which contained 2 per cent and 4 per cent gamma globulin concentrations.

FIGS. 21 to 31. Cells of strain HeLa demonstrating the *variant pinocytic* form of human serum stimulation and the degenerative form of gamma globulin stimulation.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was merely for pressure equalization during this exchange period. Lots of five to sixteen chambers were used for each experiment as determined by the number of replicate cultures available after the initial growth phase in the basic nutrient. Usually each lot was divided into three groups. One group was replenished with a fresh solution of the initiating basic nutrient, one with a benign serum nutrient(s) and one with a known cancerous serum nutrient(s). Serum nutrients were formulated following the same scheme outlined for the benign ascitic and pleural fluids. After centrifugation the sera were divided into two parts. One part was placed in the deep-freeze to be analyzed at a later date and the other part was formulated into a nutrient and exposed to the cells within three weeks of venapuncture. The results which follow were derived from experiments which used the second group of fresh serum nutrients.

Results

The *VP cell* was first noted in a malignant serum nutrient culture and was not seen in the non-malignant counterpart of the experiment. From this observation successive experiments developed, each with some distinctive feature dictated by the availability of chambers, sera and cultures adequate for analysis. Because of this, statistical values were not attempted. This first group of seven experiments which are outlined below then merely notates a cytological phenomenon which was attributed to human serum nutrients, establishes the groundwork on which quantitative techniques are being developed and reiterates the value of the living cell as an important agent for laboratory exploration.

Consecutive numbers were used for each experiment and the laboratory data book number was inserted in parenthesis. Table 1 gives pertinent data for the patients whose sera and body fluids were used in these experiments.

Experiment I (H55-5). After seven days of cultivation in the basic nutrient NAF-50 (11/24) the chambers were evacuated and replenished with the test nutrients. There were five suitable cultures at this time so serum nutrients were placed into four chambers and the fifth again received the basic nutrient. Two control serum chambers received a nutrient of serum from a thirty-six year old female (SER No. 55-3) and two chambers received a malignant serum nutrient derived from a fifty-three year old colored female with a diagnosis of squamous cell carcinoma of the vulva, grade III (SER No. 55-1). After one day of cultivation in the test nutrients there was no noted differentiation amongst the cultures. On the third day the peripheral edges of the cultures in the cancerous serum nutrient displayed a cell with large black spherical granules (B&L phase contrast) in its cytoplasm. A closer observation with the oil immersion objective (97x) demonstrated

these cells to be anatomically unchanged with other cells of this strain except for the unusual sized granules. Vacuoles of *pinocytosis* were average for peripheral cells, the limiting membrane was very active, mitochondria appeared as in the other cells and the nuclear detail was not remarkable. The chambers containing the normal serum and basic nutrients did not show this cell at this time. The chamber cultures were not observed again until an additional three days had elapsed. At this time peripheral areas of all cultures were searched and the aberrant *VP cell* was noted to be scattered generously in the malignant serum nutrient chambers and was also becoming evident to a slight degree in one of the non-malignant serum chambers. After seven days of cultivation in the sera and basic nutrients the fluids were exchanged once more. Three days later the *VP cell* dotted the peripheral edges of the cultures in the malignant serum chambers but was absent in the non-malig-

TABLE 1

Patient Data of the Sera and Body Fluids Analyzed

Serum No.	Hermann Hosp. Record No.	Patients' Initials	Age, Color and Sex	Diagnosis
SER No. 55-1	54–11615	L.T.	53 C.F.	Ca. vulva
SER No. 55-2	54–6314	I.R.	52 W.F.	Ca. endometrium
SER No. 55-3		S.H.	36 W.F.	"Normal"
SER No. 55-5	52–30485	M.McL.	71 W.F.	Ca. cervix
SER No. 55-6	49–14523	F.M.	34 C.F.	Ca. cervix
SER No. 55-7		D.P.	33 W.F.	"Normal"
SER No. 55-8		G.R.	32 W.M.	Post polio
SER No. 55-9		J.R.	31 W.F.	"Normal"
SER No. 55-10		W.M.	27 C.M.	"Normal"
SER No. 55-11	55–2297	K.W.	54 C.F.	Ca. endometrium
SER No. 55-12	51–29173	E.S.	21 C.F.	Ca. *in situ*
SER No. 55-13	54–24968	L.W.	65 W.M.	Ca. prostate
SER No. 55-14		J.G.	30 W.M.	"Normal"
SER No. 55-15	50–17614	D.R.	41 C.F.	Ca. breast
NAF-50 (11/24)				
(1) 50%	53–10131	J.T.	47 W.M.	Cirrhosis
(2) 50%	54–11982	M.K.	50 W.F.	Cirrhosis
NPF-50 (2/24)	55–29672	R.S.	53 W.M.	Congestive failure
NPF-100 (2/24)	55–29672	R.S.	53 W.M.	Congestive failure

nant serum chambers. At the end of twenty days of total cultivation (thirteen days under the influence of the test conditions) the experiment was terminated. During the final thirteen days the *VP cell* persisted and progressively increased in number and size in the cancerous serum cultures, appeared minimally and sporadically in the non-malignant serum nutrient cultures and was not observed at any time in the basic nutrient cultures.

Experiment II (H55-6). Five chamber cultures were selected after an initial cultivation period of twelve days in the basic nutrient NAF-50 (11/24). One chamber was replenished with the basic nutrient, two with a non-malignant serum nutrient derived from a thirty-six year old white female (SER No. 55-3) and two with a malignant serum nutrient derived from a fifty-

two year old white female with a diagnosis of carcinoma of the endometrium (SER No. 55-2). Within two days the *VP cell* became conspicuously present in both cancerous serum cultures and minimally so in one of the non-malignant serum cultures. The cultures were observed for only six days after the test media had been introduced. The *VP cell* again disappeared from one control culture with the non-malignant serum nutrient, persisted in the cancer serum cultures and was not evident at any time in the basic nutrient culture. The cultures under the influence of the malignant serum nutrients showed considerable degeneration along with substantial attempts toward a recovery.

Experiment III (H55-7). This brief experiment was designed to see if chick embryonic extract would excite a *VP cell* response. Two chamber cultures which had been growing for seven days in the basic nutrient NAF-50 (11/24) were evacuated and replenished with the same basic nutrient containing embryonic extract of the chick (EE-50 3 per cent and EE-25 3 per cent obtained from Microbiological Associates). These cultures were examined for seven days without observing the *VP cell.*

Experiment IV (H55-8). After seven days of cultivation in the basic nutrient NAF-50 (11/24), nine chambers were selected as test objects. Eight chambers were replenished with serum nutrients from females with cervical carcinomas (SER No. 55-5 and SER No. 55-6) and from females in the menstrual phase (SER No. 55-7 and SER No. 55-9). Two chambers were used for each serum nutrient and the ninth chamber received a fresh supply of the basic nutrient. This experiment commenced a procedure of making three notations with each microscopic observation, and these were: (1) a measurement in millimeters of the culture diameter, (2) a pH reading of the chamber fluid and (3) a count of the *VP cells* which surrounded each culture. A summary of these values was recorded in table 2. Here again the largest group of *VP cells* was found in the cultures containing the serum nutrients from malignant hosts. On the eighteenth day the three remaining chambers which contained serum nutrients were evacuated and replaced with the basic nutrient. The chamber which had contained the basic nutrient throughout the first eighteen days was evacuated and replenished with a malignant serum nutrient (SER No. 55-6). After three days the *VP cells* had completely disappeared from the chambers containing the basic nutrient but were commencing to appear in the chamber containing the malignant serum nutrient. This last procedure demonstrated the reversible nature of this cytological response.

Experiment V (H55-9). Four chambers which had been cultivated for thirteen days in the basic nutrient NAF-50 (11/24) were selected as test objects to evaluate the *VP* response potential in gamma globulin. Two chambers were evacuated and resupplied with the basic nutrient containing pooled gamma globulin 2 per cent and two chambers were resupplied with pooled gamma globulin 4 per cent. After two days of cultivation under this influence a detectable cytological change could not be made. On the third day, however, each chamber revealed cells undergoing a degenerative cellular reaction which was characterized by globulations of the limiting cell

TABLE 2

Factual Data Obtained From Experiment IV

Chamber No.	SER No.	7 Days pH	Diam	10 Days pH	Diam	VP	12 Days pH	Diam	VP	17 Days pH	Diam	VP
2	55-7	7.5	4.0	7.5	4.0	2						
3	55-7	7.5	4.0	7.5	4.0	3						
8	55-5	7.4	4.0	7.0	5.0	8	6.8	5.0	18	6.9	5.5	20
10	55-5	7.4	4.0	7.0	4.3	5						
11	55-6	7.5	4.0	7.1	5.0	5	7.1	5.0	6	7.0	6.0	16
21	55-6	7.5	4.0	7.3	4.5	5						
23	55-9	7.5	4.0	7.2	4.5	2	7.4	4.5	5	7.3	4.5	9
14	55-9	7.5	4.0	7.3	4.8	2						
15	NAF-50 (11/24)	7.8	4.0	7.8	4.0	0	7.8	5.0	0	7.7	5.0	0

SER No.—This refers to the particular fluid nutrient listed in table 1.
pH—The pH was determined by comparing the culture chambers with chambers containing standard buffer mixtures.
Diam—The diameter of the culture expressed in millimeters.
VP—The number of *variant pinocytes (VP cells)* per culture.

membrane (Figs. 27–31). These globulations often appeared as a cluster of grapes (Fig. 30). The fourth day demonstrated an almost complete degeneration in one of the 4 per cent chambers; the 2 per cent chambers showed areas of degeneration contrasted by other areas which were apparently unaffected. The degenerating cells eventually became refractile spheres. After seven days the 2 per cent cultures showed less than 25 per cent viable cells. The production of this characteristic degenerative pattern apparently was a direct response to a property of gamma globulin. Cells degenerating in this fashion have been seen in HeLa cultures growing in various other media but as such were always isolated. The degenerating cell under the influence of gamma globulin occurred in composite areas and eventually involved the entire culture.

Experiment VI (H55-10). Sixteen chamber cultures which were essentially replicate after ten days of cultivation in the basic nutrient NAF-50 (11/24) were evacuated and replenished with eight pairs of various nutrients. The nutrients and the results obtained were summarized in table 3. Note here that (1) SER No. 55-5 from a patient with a cervical carcinoma produced a large supply of *VP cells* as in *Experiment IV*, (2) SER No. 55-7 from the menstruating female gave an immediate high response and then waned rapidly as before and (3) SER No. 55-9 also from a menstruating female compared favorably with the former data. Note also that the 100 per cent pleural fluid NPF-100 (2/24) produced the *VP cells* whereas a 50 per cent concentration NPF-50 (2/24) failed to demonstrate them, old pooled serum nutrients showed a weak response and human embryonic extract supported some *VP cells* whereas formerly chicken embryonic extract failed produce them.

Experiment VII (H55-13). Eight chamber cultures were selected at twelve days of cultivation in the basic nutrient NAF-50 (11/24) and eight conditions were evaluated. The results were summarized in table 4. The rise of

TABLE 3

Factual Data Obtained From Experiment VI

Chamber No.	SER No.	10 Days pH	Diam	11 Days pH	VP	13 Days pH	Diam	VP	14 Days pH	Diam	VP
24	55-5	7.4	5.0	7.4	15	7.0	7.0	22	7.0	7.0	12
1	55-5	7.4	5.0	7.4	30	7.0	7.0	27	7.0	7.0	10
25	55-9	7.6	5.0	7.5	6	7.2	7.0	3	7.2	7.0	0
16	55-9	7.6	5.0	7.5	7	7.1	8.0	8	7.1	7.5	7
20	55-7	7.6	5.0	7.5	22	7.2	6.0	9	7.2	6.5	2
30	55-7	7.6	5.0	7.5	25	7.2	7.0	10	7.2	7.0	7
3	NAF-50 Hum. EE	8.1	5.0	7.7	4	7.6	6.0	0	7.6	6.0	2
12	*ditto*	8.1	5.0	7.7	1	7.6	6.0	0	7.5	6.0	0
6	NPF-50	7.4	5.0	7.2	0	7.1	7.5	0	7.0	7.5	0
13	NPF-50	7.4	5.0	7.2	0	7.1	6.0	0	7.0	6.5	0
4	NPF-100	7.4	5.0	7.2	3	7.1	5.0	5	7.0	5.0	2
7	NPF-100	7.4	5.0	7.2	4						
2	Hum. ser. pooled.	7.3	5.0	7.2	3	7.1	7.0	2	7.0	6.5	0
9	*ditto*	7.3	5.0	7.2	2	7.1	7.0	1	7.0	6.5	0
14	NAF-50	8.2	5.0	7.9	0	7.5	7.0	0	7.4	7.0	0
10	NAF-50	8.2	5.0	7.9	0	7.5	7.0	0	7.4	7.0	0

SER No.—This refers to the particular fluid nutrient listed in table 1.
pH—The pH was determined by comparing the culture chambers with chambers containing standard buffe mixtures.
Diam—The diameter of the culture expressed in millimeters.
VP—The number of *variant pinocytes (VP cells)* per culture.

VP cells in the two serum nutrients SER No. 55-11 and SER No. 55-15, which were from female patients with endometrial and breast carcinomas, presented a striking curve. This is the first experiment in which *VP cells* were sought at such an early interval following serum nutrient additions (three hours). There was an immediate response in most chambers but these early values did not parallel repeated interval counts of the subsequent four days. This was also the first experimentation involving a male malignant serum nutrient and it was interesting to note that there was no appreciable rise in the number of *VP cells* stimulated. The carcinoma *in situ* was also interesting from the standpoint that it too showed no abundant production of the sought cell. The *VP cell* predominantly was seen in the peripheral edges of the cultures. An 8 mm. diameter culture would contain almost 40 per cent more circumference than a 6 mm. diameter culture. Because of this understanding the intermediate elevation of *VP cells* in the healthy post polio male would probably have been appreciably lower in a smaller culture.

Discussion. The results presented above represent a total observation of forty-nine HeLa cultures as viewed over a varying period of time in multipurpose culture chambers through phase contrast optics. Fourteen sera, eight of which were from known cancer-bearing human hosts, one benign pleural fluid, two benign ascitic fluids, old pooled human serum nutrient, human and

TABLE 4

Factual Data Obtained From Experiment VII

Chamber No.	12 Days SER No.	(3 hrs.) pH	Diam	VP	(20 hrs.) pH	VP	(28) VP	(48 hrs.) pH	Diam	VP	(72 hrs.) pH	Diam	VP	(96 hrs.) pH	Diam	VP
25	55-14	7.6	6.0	0	7.4	2	4	7.0	6.5	6	7.0	8.0	6	6.9	8.0	8
3	55-11	7.6	5.0	5	7.6	7	18	7.3	6.5	16	7.3	6.5	19	7.2	7.0	32
28	55-12	7.8	5.0	3	7.6	5	4	7.2	7.0	4	7.2	7.0	3	7.0	7.5	9
5	55-8	7.6	6.0	11	7.4	15	18	7.2	7.0	20	7.0	8.0	22	7.0	8.0	15
6	55-10	7.4	5.0	3	7.4	4	6	7.2	5.5	4	7.1	6.0	10	7.1	6.0	2
16	NPF-50	8.0	5.0	0	7.8	0	0	7.7	6.0	0	7.7	7.0	3	7.6	7.0	3
24	55-15	7.2	5.0	1	7.2	4	8	7.2	5.5	25	7.1	6.0	32	7.1	6.0	31
1	55-13	7.4	5.5	0	7.4	2	3	7.3	6.0	7	7.2	7.0	6	7.2	7.0	9

SER No.—This refers to the particular fluid nutrient listed in table 1.
pH—The pH was determined by comparing the culture chambers with chambers containing standard buffer mixtures and phenol red.
Diam—The diameter of the culture expressed in millimeters.
VP—The number of *variant pinocytes (VP cells)* per culture.

chicken embryonic extracts and gamma globulin additions were screened for their effect on the *VP cell* production. These results were merely an unusual finding which may or may not bear directly upon the solution of *factor X*. The occasional disappearance of this cell entity from "normal" serum nutrient cultures suggested that the pinocyte had removed all of the ingredient from the nutrient. Because the *VP cells* eventually became dying systems in a stuffed bag form they were similar to phagocytes which often overload their physical means of capacity.

The series of events: (1) active pinocytosis, (2) juxtanuclear granule enlargement and (3) granular bag consummation with eventual cell death, advanced the idea that the pinocyte reacted to a stress condition which was not enjoyed by all of the cells. Its reaction to the stress resulted in a *variant pinocytic cell (VP cell)*, a buffering cell which removed the stress condition at its own expense.

The reversibility of the *VP cell* reaction substantiated this to be a true response to the bathing serum nutrient. The fact that old pooled serum which contained 50 per cent known malignant serum proved such a poor stimulus suggested that best results should come from freshly drawn specimens. The presence of a human embryonic extract stimulation and the absence of a like stimulation from chicken embryonic extract humanized this reaction. When the pleural fluid was used in 100 per cent concentrations a response similar to the normal serum nutrients was ob-

served; whereas, at 50 per cent concentrations the *VP cell* was only demonstrated in one of three chambers and this was slow to develop. Since transudates are less than 4 per cent protein, serum nutrients diluted to a 50 per cent solution with balanced salt solution would then contain a closer protein level to the 100 per cent pleural fluid. This may be true for other components. I do not mean to infer that serum proteins are the responsible agent, but merely to point out that a 100 per cent pleural fluid nutrient should more closely approximate a 50 per cent serum nutrient than would a 50 per cent pleural fluid nutrient.

Without biochemical analyses at this time we can merely speculate etiological factors. Histochemical and cytochemical staining may indicate the nature of the granules. Attempting to add part by part fractions of serum to non-producing ascitic fluid nutrients in an effort to elicit the *VP cell* response is an intriguing prospect. A relationship of this cell type to pregnancy, menstrual phases, preoperative and postoperative states as well as to the known clinical courses of chronic and fulminant diseases with or without treatment is a demanding objective. A program has commenced which will permit statistical evaluation so that the sera which heavily stimulate this cell may be investigated with regard to why they do so and from what group of patients they are derived. In time other cell types will be used in an attempt to bridge the gaps of sex, tumours and other variables which may appear to influence this cytological reaction.

It is felt that these results present enough evidence to continue pursuit in this direction of cellular sensitivity and that by subjecting enough sera to enough replicate cultures, biological quantitative evaluation may seriously challenge the more prosaic methods of seeking "*factor X*" in sera from cancer patients.

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IMMUNOLOGIC STUDIES IN HODGKIN'S DISEASE*

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WITH THE TECHNICAL ASSISTANCE OF
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The idea of search for a specific antigen in Hodgkin's disease stemmed from the possibility that lymph node and reticuloendothelial tissue probably lacks the high degree of specificity possessed by more highly organized and uniform cell population type of tissue such as epithelium. The presence of macrophages or wandering cells that may have altered antigenic properties because of the influence of various hormonal or enzymatic factors present locally in other tissue or organs of the body in which they may have sojourned would contribute to this heterogeneity.

Because of the lesser degree of specificity the potentiality would be likely to be increased for the development in the presence of irritants, metabolic waste products, and phagocytosed substances, of an antigen "foreign" to the individual. Continued stimulation of the cells in a lymph node draining a particular area subject to prolonged irritations is postulated to result in an hyperplastic reaction and finally to the exhaustion of cell types such as the reticulum cell of the involved lymph node.

Green (1954) in his immunologic hypothesis of carcinogenesis states that various hydrocarbons have been demonstrated to produce an immune response in the regional lymph nodes of animals. Further he believes that carcinogenic hydrocarbons have resulted in a greater degree of hyperplasia than that associated with the immune reaction to non-carcinogenic hydrocarbons. Green suggests that cells subject to such continued hyperplasia eventually become exhausted and fail to synthesize one or more

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of the proteins normally characteristic of this cell, "identity proteins." These cells, he suggests, have undergone the first step toward neoplasia since the "marker proteins" have been lost and thus, according to Burnet and Fenner (1949), no antibody formation will be stimulated since the "recognition units" are lost.

Infectious agents have been variously incriminated as etiological factors in Hodgkin's disease. The possible etiological significance of bacteria and viruses found in Hodgkin's diseased lymph nodes is extremely hard to evaluate. Whether they may represent non-specific factors that serve as irritants to initiate a neoplastic type reaction or whether one of the many suspected agents may be an actual causative agent is problematic. Yates (1915) and Yates and Bunting (1915) reported that they had demonstrated a gram positive pleomorphic diptheroid organism in pure culture in a predominant number of glands obtained immediately after surgical excision. Bostick (1952) has been a strong proponent of the virus etiology of Hodgkin's disease based on his findings in tissue cultures. Reagan (1954) demonstrated virus-like particles in red blood cells from Hodgkin's disease under the electron microscope, but was unable to demonstrate them in the red cells from normal patients. Frajola and his associates (1955) warn that these particles seen in electron microscopy may be artifacts.

Studies in Hodgkin's disease based on an hypothesis of immunologic pathogenesis have been conducted over the past four years. A summary of the various aspects of this study are presented below. The basic approaches have attempted to answer the following specific questions: (1) Whether tissues affected by Hodgkin's disease when injected into rabbits are capable of provoking the formation of specific antibodies; (2) whether such antibodies when injected into the patient with Hodgkin's disease can be demonstrated to penetrate the cells affected by this disease; (3) whether such antibodies localized in vivo, in the diseased cells, will have a favorable influence on the course of the disease.

The approaches that have been employed include the following: (1) rabbit immunizations; (2) conventional immunologic methods of titering antisera; (3) evaluation of tissue localizing characteristics of antisera in tissue cultures; (4) evaluation of

tissue localizing characteristics of antisera in the patient with Hodgkin's disease; and (5) studies to determine whether injection of Hodgkin's antigen preparations in patients affected with this disease would provoke formation of antibodies.

Rabbit Immunizations. Tissue was obtained from Hodgkin's diseased patients either at biopsy or at post-mortem. Most post-mortem material was removed within four hours of the patient's death. All tissues were stored in the presence of carbon dioxide. Normal lymph node tissue for control immunizations and titering was obtained by the same methods as described for Hodgkin's tissue. Patients with diseases affecting the lymph nodes, either primarily or secondarily were not used as sources of normal lymph node antigen.

While various types of immunization preparations and fractions of Hodgkin's tissue were employed in the many immunization programs conducted over the past four years, two techniques have been successful in production of antisera which gave evidence of specificity against Hodgkin's antigens, as evidenced by complement fixation reactions employing the 50% hemolysis unit. Use of the whole homogenate with adjuvants consisting of the non-pathogenic organisms, myco-bacterium phlei, falba, and mineral oil produced evidence of a differential titer against Hodgkin's antigen as compared to the titer against normal lymph node antigen. This group of animals was immunized at weekly intervals for twelve weeks. No evidence of a specific titer was noted until eight months after the last injection had been given. During a two-month period following the initial finding, the specific titer persisted, Tables 1 and 2).

A second group of animals were immunized on a schedule of 0.05 cc., 0.1 cc., 0.2 cc., and 0.4 cc. of antigen intravenously on the first four days of each of three successive weeks with the fraction of Hodgkin's tissue soluble after 30 minutes centrifugation at 2000 r.p.m., but not soluble at 20,000 r.p.m. for 10 minutes, showed an early differential titer against Hodgkin's disease which disappeared approximately two months after immunization was stopped. After a rest period of a few months, booster injections of this material again renewed the differential titer. Failure to reproduce these results in a second group of animals, on analysis, has been shown to be quantitative rather than qualitative. Thus,

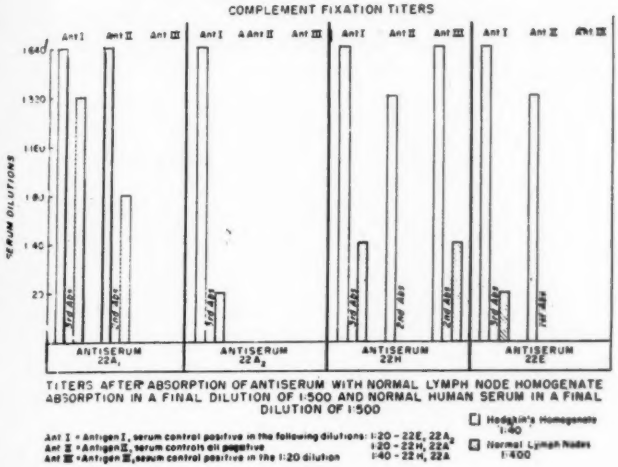


TABLE 1

differences were shown to exist in the sera of some of this second group which had previously failed to show any differential titer on use of smaller quantities of complement in the quantitative complement fixation reaction. One possible explanation of the difficulty may be changes in the antigen during prolonged storage, and the influence of the summer heat on the titer of the second group of animals.

Studies of the Hodgkin's antigen. From the time the first evidence of specificity of some component of the Hodgkin's antigen was obtained in immunization of rabbits with a whole homogenate preparation, methods of purification of the Hodgkin's antigen have been in progress. These approaches have been directed chiefly toward purification of the antigen used for immunization. Antigen sources have been sought from both Hodgkin's granuloma type of lesion and tissue completely replaced by tumor-like masses of Hodgkin's sarcoma cells since either type of tissue is likely to contain the altered antigen which represents the change to neoplasia compatible with the proposed immunologic hypothesis outlined above.

Preliminary studies in the ultracentrifuge, using a modified Stern apparatus with a direct Schleriren system, and a few

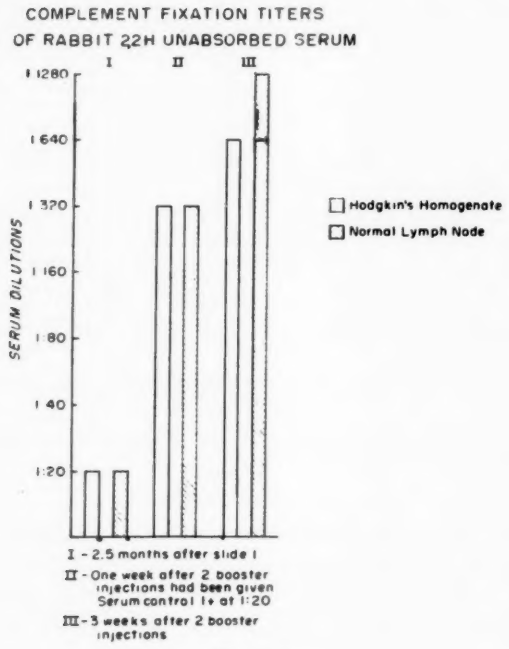
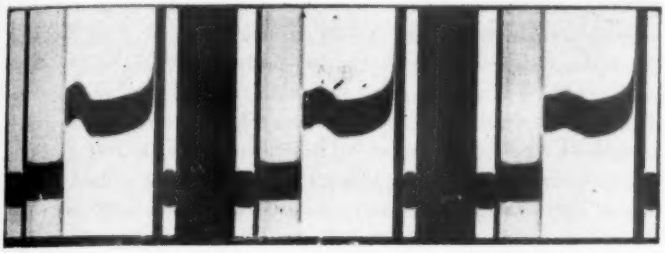


TABLE 2

studies done in the Model "E" Spinco instrument (Fig. I) suggest a characteristic peak present in Hodgkin's disease, but not present in the normal lymph node specimens subected to analysis. Chromatographic studies have been done on homogenates of tissues of patients with Hodgkin's disease following injection of the tagged rabbit anti-Hodgkin's globulin.

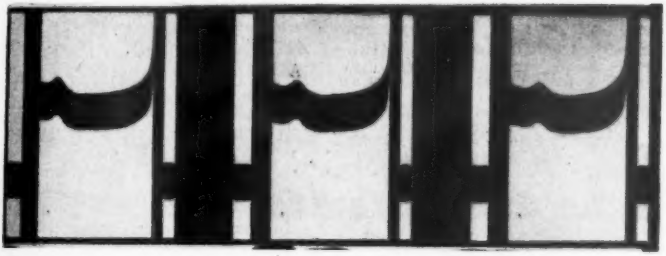
Antibody Purification. To date, two main techniques have been employed for purification of the rabbit anti-Hodgkin's serum. Serial absorption of anti-bodies against normal lymph node and normal human serum preceded experiments in which the globulin fraction of the antiserum was tagged with radioactive iodine. In the antisera obtained from the animals immunized with the whole homogenate preparation and showing a specific titer against Hodgkin's, additions of a whole homogenate of normal lymph node followed by continuous rotation

Sample Number RTC 1
Speed: 59,780
Temperature: 23.9° C.
Hodgkin's Diseased Lymph Node



Frame No. 11 Time 44 min. | Frame No. 12 Time 52 min. | Frame No.13 Time 60 min.

Sample Number RTC 2
Speed: 59,780
Temperature: 22.3° C.
Normal Lymph Node



Frame No. 11 Time 60 min. | Frame No. 12 Time 68 min. | Frame No. 13 Time 76 min.

FIG. I. Hodgkin's lymph node component B found in other Hodgkin's diseased tissues, but not found in any normal lymph node specimen.

in the cold overnight and centrifugation at 2500 r.p.m. for 30 minutes resulted in disappearance or diminution of the titer against normal lymph node in two to three such serial absorptions. When no differential in titer or when only a slight difference existed, much more difficulty was encountered in showing any evidence of absorption of antibodies against normal lymph node by the techniques described above. Whether this was due to the actual lack of specific antibodies against Hodgkin's

antigen or due to the small amount of antibody that was specific for Hodgkin's as compared to the much larger amount of various antibodies against the different lymph node components has to date not been determined.

In experiments in which ultracentrifugation in the Model "E" Spinco followed the absorption procedure there was a little more evidence of success with the same types of preparations indicating that some removal of the more soluble antigen antibody complexes was accomplished by this technique. Another complicating factor exists in the absorption methods described above. When only small quantities of any particular antibody are present, the addition of more antigen for performance of complement fixation experiments shifts the balance further in the region of antigen excess, and thus results in additional masking of the specific antibody. Complement fixation reactions employing various quantities of complement lent support to the supposition that the antibodies for a specific Hodgkin's antigen are overshadowed by the much greater quantities of antibodies against other antigens present in the immunization preparation. In studies of many sera that failed to show a specific titer with Hodgkin's antigen preparations employing four units of complement, experiments were done with six, four, and three units of complement. No differential in titer existed in comparing the titers with Hodgkin's antigen and with normal lymph node antigen, but at one and a half units of complement using the 50% unit of hemolysis, differences were demonstrated for most of the antisera obtained following immunization with the fraction of Hodgkin's lymph node that was soluble between 3000 r.p.m. and 18,000 r.p.m. in the ultracentrifuge. Rapport (personal communication) has shown evidence of this same phenomena in his work. He was able to remove the soluble, or semi-soluble complexes by ultra-centrifugation as described above.

The globulin fraction of the antiserum has been prepared by dialysis of these sera against 1.4 vols. of 3 m. ammonium sulphate continuosuly rotated in the cold overnight. The precipitate obtained was washed several times with 1.75 m. ammonium sulphate and reconstituted to it's original volume of serum in normal saline.

Because of the difficulties described above in absorption and quantitative complement fixation reactions along with the large number of non-specific gammaglobulins of the rabbits that are tagged with the radioactive iodine at the same time that the rabbit anti-Hodgkin's globulin is being tagged, other methods of purification are being employed. (Talmage, Baker and Akeson, 1954.)

These techniques involve absorption of specific antibodies by tissue slices of Hodgkin's disease followed by elution with carrier globulin and citric acid, according to the techniques described by Talmadge and Maurer (1953).

In Vitro Tissue Localizing Antibodies. Tissue cultures of Hodgkin's diseased lymph nodes were prepared from post-mortem tissues less than four hours old, surgical specimens, and tissues from either of these sources that had been stored at temperatures below —40° C. Explants were set in clots consisting of equal parts of chick embryonic extract, Tyrode's solution and unheparinized rooster plasma. Supernatant fluid consisted of equal parts of human cord serum or ascitic fluid, Tyrode's and beef serum ultra-filtrate. Roller tube, Maximow slide and hanging drop preparations were employed in this study.

Lack of evidence of viability of the Sternberg-Reed cells in the growing sheet of cells in cultures other than those obtained from fresh biopsy specimens, has prevented a regular program of study, up to the present time, of living Hodgkin's cells in tissue cultures. The use of 0.5 percent methylene blue as a supra-vital stain has been effective in demonstrating viability of the components of the tissue culture.

The effect of addition of a rabbit anti-Hodgkin's serum on tissue cultures of Hodgkin's diseased lymph nodes was observed in a few experiments employing surgical specimens of Hodgkin's diseased lymph nodes. Following a control observation period of this type of culture in a perfusion chamber setup, under the phase contrast microscope, additions were made of normal rabbit serum and later of rabbit anti-Hodgkin's serum.

Cinematographic sequences were made over a period of 24-48 hours at regular intervals employing time lapse equipment attached to a 16 mm. Bolex Deluxe Camera. Destructive effects on the Sternberg-Reed type cell were observed to occur different-

ially. A similar effect was not noted in other components of the outgrowing sheet. Rabbit normal serum and rabbit anti-normal lymph node serum did not produce this result. Studies of cytotoxic effects in tissue culture parallel the work of Pomerat and Anigstein (1945) using a rabbit anti-spleen serum.

In Vivo Studies. The globulin fraction of a rabbit anti-Hodgkin's serum was tagged with I^{131} in tracer amounts and injected in patients with Hodgkin's disease. Antibodies against normal lymph node and normal human serum were previously removed by serial absorption with these antigens. Control studies in which the patient was injected with rabbit globulin tagged in a similar fashion were performed. Uptake of the radioactive material over the various parts of the body was measured with a scintillation type counter. Counts over involved lymph nodes were compared with those made over the corresponding uninvolved area on the contra-lateral side of the body. Blood disappearance curves and appearance of radioactivity in the urine were determined. Counts were performed on biopsy specimens and on post-mortem specimens to determine whether localization was specific for tissue showing involvement by the Hodgkin's disease process. Microscopic autoradiographs were prepared (Fig. 2) from biopsy and post-mortem specimens in three of these patients. Lantern slides and stripping film type preparations were prepared of normal lymph nodes and Hodgkin's diseased nodes. When other tissues were available, autoradiographs were made from normal and Hodgkin's infiltrated sections of these tissues.

To date, six patients have been studied employing varying number of the type determinations enumerated above. The patient's condition, number of involved areas and degree of cooperation determined the completeness of any individual study. The evidence to date from microscopic autoradiographs seems to indicate specific uptake of the rabbit anti-Hodgkin's globulin by the histologically abnormal cells in the Hodgkin's diseased tissues.

Masking of results by many interfering factors have been responsible for inability to obtain conclusive results in some areas of the study. The minute volume of blood coursing through the arteries contains a relatively large percentage of radioactivity

in comparison to the amount localized in the external lymph nodes lying in close approximation to these vessels.

In two studies, a normal and an Hodgkin's diseased lymph node, as well as samples of other tissues and organs were obtained following injection of tagged normal rabbit globulin and tagged globulin from a rabbit immunized with an Hodgkin's antigen

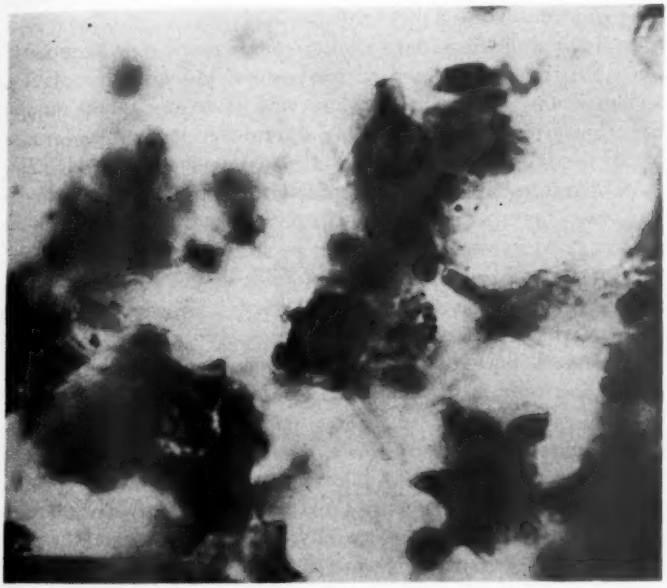


FIGURE II

preparation. Since two distinguishable isotopes were not employed for tagging the sera, a two stage procedure of injection and removal of tissue would be required in order to have an adequately controlled study. In the most recent patient study a Hodgkin's diseased node was obtained following injection of the globulin fraction of normal rabbit serum tagged with I^{131}. A normal node could not be obtained at this time. Following injection of a rabbit anti-Hodgkin's serum, however, both a normal and several Hodgkin's diseased lymph nodes were obtained.

Other interfering features include large deep seated masses

of Hodgkin's diseased tissue as well as multiple areas of involvement. The resulting wider dissemination of tagged molecules increases the total required number of tagged antibody molecules. A resultant increase of non-specific activity is introduced thereby. Others working with much purer antigens than can be prepared from tissue employed in this study, report that less than 3 percent of the radioactivity is attached to the antibody globulin used in immunization of the animal.

In three of the first four patients studied no tissue was obtained for study by autoradiography or counts of emitted radiation. Evidence for specific localization was based on uptake studies over the various areas of the body and rate of blood disappearance of radioactivity as compared with tagged normal rabbit globulin. A photomicrograph of an autoradiograph of a cervical node obtained at surgery from the fourth patient is illustrated in figure II.

In the past two patients studied analysis has been more complete. All of the available tissues were homogenized and the radioactivity associated with protein, polypeptides and amino acids and to inorganic halides have been determined. The partition of activity in the blood was similarly determined.

Whole tissues were washed with saline, 0.05 percent Potassium Iodide and normal human serum to remove any blood or serum present in the tissue. The homogenized tissue was centrifuged at 3000 r.p.m. for 5 minutes, and subsequently for 90 minutes to separate microsomal and mitrochondrial fractions. The partition of radioactivity was determined between these various fractions. Subtraction of activity due to presence of blood and tissue fluids left undisturbed the ratio of radioactivity in Hodgkin's diseased lymph nodes and normal lymph nodes. Hodgkin's diseased lymph nodes contained significantly less localized radioactivity following injection of tagged normal rabbit globulin than following injection of the rabbit anti-Hodgkin's globulin even without purification of the rabbit anti-Hodgkin's globulin.

Analysis of autoradiographs from these past two studies are still in process. However, the present trend appears to indicate specific localization in the Hodgkin's diseased cells.

In the fifth patient study samples of all of the major tissues were obtained at post-mortem. While the data from the counts of

activity in the various tissues showed a positive correlation with pathologic involvement with Hodgkin's disease the data was not statistically significant because of the proximity of the counts in the tissue to the background counts.

Shrinkage of peripheral lymph nodes to approximately one-fifth the original size occurred within thirty-six hours after administration of 60 cc. of rabbit anti-Hodgkin's serum tagged with 8 millicuries of radioactive I^{131}. This patient was terminal. Since the patient was comatose and the respirations gasping in character, supportive therapy had been stopped. Within eight hours after administration of antiserum, the patient began to respond and supportive therapy was started again. The patient regained consciousness, was able to take nourishment and became lucid. However, the trend reversed itself in six days. Additional radio-active antiserum was available only in minute quantities. The patient expired on the seventh day. At post-mortem the patient showed a diffuse Hodgkin's sarcoma with large tumor masses and extensive replacement of the various organs of the body by Hodgkin's involvement. In the sixth patient studied, tissue removed after injection of I^{131} tagged normal rabbit globulin emitted 6,047 counts [minute] gram of tissue. After injection of I^{131} tagged rabbit anti-Hodgkin's globulin a normal lymph node contained 17,600 counts [minute] gram of tissue. Hodgin's diseased nodes contained from 27,750 counts to 47,700 counts [minute] gram of tissue with an average of 36,000 counts [minute] gram. After absorption with normal rabbit globulin and potassium iodine, the ratio of the counts were maintained as before absorption.

Patient Response to Hodgkin's Antigen Administration. Presence of a specific antigen in Hodgkin's disease would be expected to stimulate an antibody response. However, if the altered lymph node cells vary sufficiently, antigenically, from the normal cells and lack "recognition units," no antibody response is likely. In addition, cells that vary sufficiently in antigenic specificity from the normal cell population of any particular tissue may not elicit an antibody response, unless removed to another area of the body.

On the hypothesis that introduction of cells in various stages of development from normal to the fully developed Hodgkin's

diseased cells into the body intradermally, subcutaneously or intravenously might result in production of antibodies to antigens not normally present in the patient, the following studies were done.

Five patients with generalized Hodgkin's disease were treated with Hodgkin's homogenate supernates absorbed on aluminum hydroxide. Three of these patients were in advanced stages of Hodgkin's sarcoma, two had generalized involvement with Hodgkin's granuloma.

Evidence for development of antibodies not normally present in normal human serum was obtained in the three patients on whom determinations were performed. Two of these patients had sarcomatous type of disease and the other granulomatous. After several months of antigen therapy, serum from these patients was obtained and injected into guinea pigs. Seventy-two hours later, intravenous injection of the antigen into the guinea pigs produced grossly detectable anaphylactic reactions in the lungs of all sensitized animals. This did not occur in animals previously sensitized with normal human serum or serum from untreated Hodgkin's patients.

After a minimum length of therapy of three months, increase in size of an individual group of peripheral lymph nodes associated with marked tenderness and pain occurred, in three of the patients under therapy. This local phenomen was noted in spite of marked general improvement of the patient. From both a diagnostic and therapeutic standpoint a group of inguinal lymph nodes were removed from one of the patients with Hodgkin's granuloma. Pathologic examination revealed marked necrosis. "Ghost cells" were present and appeared to represent the remainder of Sternberg-Reed type cells. The only intact cells that remained were lymphocytes. This same phenomenon appeared to be taking place in the other patient in this series with Hodgkin's granuloma. However, before a biopsy could be done a group of axillary nodes became suppurative, discharged to the outside and subsequently revolved completely. A similar phenomenon in one of the patients with an Hodgkin's sarcoma was also associated with general improvement. Examination of the biopsy specimen, while following the same general thesis as that noted in the first patient, did not show the marked "ghost cells" occurrence and

the absence of all components other than lymphocytes. However, a marked lymphocytic hyperplasia was noted.

Lymphocytic hyperplasia, noted above, suggested the possibility of return of radiosensitivity in the far advanced patients who were no longer radiosensitive. Marked clinical improvement was noted in all five patients in this series who, following several months of antigen therapy, were subsequently irradiated.

Discussion. Evidence for specificity of an antigen occurring in Hodgkin's diseased tissue has been presented. The results of the multiple lines of approach to this problem are not represented as conclusive of such specificity. However, the evidence obtained from complement fixation studies on the rabbit immune serum, studies of differential localization of the tagged antibody in Hodgkin's diseased tissue following intravenous injection of the antiserum into the patient and the evidence for stimulation of antibody formation following multiple injections of antigen into the patient suffering from Hodgkin's disease all point to the likelihood that an important therapeutic approach to Hodgkin's disease has been brought to light. A calculated risk was taken that the many types of experiments employed might have led to sufficient dilution of effort that nothing experimentally significant would have resulted.

However, this method was chosen rather than bogging down with a single method of approach that appeared to have yielded to the solution of the problem the maximum amount likely from that particular approach. Thus, complement fixation reactions have shown evidence of a differential titer against an Hodgkin's antigen in rabbit's immunized with a preparation containing a whole homogenate of Hodgkin's disease lymph node with adjuvants. Accordingly, this method has been employed in assessing results of animal immunization. However, in evaluation of sera that showed no differential in titer many complex problems arose. The various possibilities that existed, as discussed under "Antibody Purification" could only be analyzed by combination of techniques that were fraught with pitfalls of interpretation in addition to the extremely numerous and prolonged determinations that would be involved. Use of techniques involving assay for localizing antibodies "in vitro" and "in vivo" appeared to offer a method of analysis which was directly in line

Antigen Therapy of Patients with Hodgkin's Disease

	EG	EC	WB	TH	VS
Length of Antigen Therapy	6 weeks	2½ years	10 months	5 months	10 months
Type of Disease*	H.S.	H.G.	H.S.	H.S.	H.G.
Response	Moderate improvement under treatment	Excellent	Good for five months, then failed	Moderate during time under treatment	Good while under treatment and has continued for ten months after stopping treatment
Ultimate Outcome	Died three months after treatment stopped	General involvement disappeared	Response ceased after five months Pt. expired	Died five months after treatment was stopped	Continued to do well ten months after stopping treatment
Stage of Disease When Therapy was Started	General marked cachexia, very far advanced	Generalized, Nutrition fairly good, No anemia	Systemic involvement, fever, weight loss, anorexia	Generalized weight loss, weakness, marked anemia, anorexia	Pulmonary vertebral and multiple peripheral node involvement, moderate weight loss
Increase and Tenderness in a group of external lymph nodes	0	4, 6	6	2, 6	5, 6
Evidence of antibodies in studies of passive sensitization of guinea pigs.	+	+	+	Not done	Not done

* H.S.—Hodgkin's sarcoma H.G.—Hodgkin's granuloma

Key for changes in lymph nodes:
1. Increased lymphocytosis.
2. Diminution in number of abnormal cells in addition to 1.
3. Appearance of "ghost cells" of Sternberg-Reed type cell in addition to 1 and 2.
4. Marked general necrosis in addition to findings of 1, 2, and 3.
5. Suppuration followed by discharge of necrotic material and subsequent healing with disappearance of nodes in that area.
6. Renewal of radiosensitivity after antigen therapy.

with the goals of the study and at the same time avoided these pitfalls. Evidence of specificity employing the complement fixation relation, was next obtained employing a somewhat more refined ultracentrifugal fraction prepared from Hodgkin's diseased tissue. Thus, the approach employing purification of the antigen and the resultant antibody appear to offer the most direct attack on the specific questions set forth in the introduction. Individual treatment of the major approaches will be given in separate presentations in the future. In experiments such as the patient studies with the tagged antiserum, the fully developed experimental design has come about only after several experiments. However, additional work on antibody purification by methods such as these described by Talmadge, and Eisen (1949) will probably enhance antibody purification. Lastly, the therapeutic possibilities of this approach will constantly be kept in focus.

Attack on the origin of a specific antigen in Hodgkin's disease must await completion of the studies outlined. Rare iso-antigens, antigens occurring in other diseases of lymph nodes, increase in factors present in normal lymph nodes in infinitesimally small quantities, but in large amounts in Hodgkin's disease are all possibilities that would require consideration.

Summary. Results of complement fixation tests on the serum of rabbits immunized with Hodgkin's tissue components have been reported. Preliminary evidence has been obtained in tissue culture of a specific cytotoxic effect of this rabbit anti-Hodgkin's serum on the Sternberg-Reed type of cell.

Evidence of specific localization in Hodgkin's diseased tissue has resulted from injection of I^{131} tagged with rabbit anti-Hodgkin's serum in Hodgkin's patients.

Clinical studies employing an Hodgkin's antigen for injection into patients has shown interesting therapeutic effects.

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THE PANTOTHENIC ACID DEFICIENT RAT. II. FURTHER INVESTIGATION OF ACETATE-1-C^{14} METABOLISM*

LELAND L. SMITH† AND ROY B. MEFFERD, JR.

In a previous communication (Smith and Mefferd, 1955) the rate of incorporation of acetate-1-C^{14} into cholesterol by rats deficient in pantothenic acid (PA) was described. Tracer doses of labeled acetate injected intraperitoneally (i.p.) resulted in a more rapid uptake of the isotopic carbon into cholesterol by the intact rat deficient in PA than by control animals. This report is a continuation of the previous communication, in which lipogenesis and carbon dioxide expiration are examined.

CO_2 Expiration Experiments. Young male Sherman-derived rats were made PA deficient as described earlier (Smith and Mefferd, 1955). All details of feeding and handling were the same as described in that report; the controls were pair-fed with the deficient animals. As soon as the animals developed detectable symptoms of the deficiency (acute symptoms were not allowed to develop), all animals were injected i.p. with 6.0×10^7 counts/minute/100 g. of body weight of sodium acetate-1-C^{14} (prepared from barium carbonate—C^{14}). Immediately following the injection the rat was confined in a metabolism chamber and CO_2-free air was drawn through the apparatus at a constant rate. The air was passed progressively through 20 per cent sodium hydroxide solution, saturated barium hydroxide solution, and saturated sodium chloride solution (Skipper, *et al.*, 1948), and then into the chamber. Expired air was passed into 20 per cent sodium hyroxide solution (prepared from CO_2-free distilled water) contained in 80 cm. columns equipped with sintered glass gas dispersion disks in their ends. Expired gases could be passed through alternate columns of alkali without interruption of the system.

The sodium carbonate-sodium hydroxide solution was precipitated with saturated barium chloride solution; the barium carbonate-C^{14} was filtered, washed, dried, weighed, and assayed as an infinitely thick layer. The specific activity was calculated according to the methods of Van Bruggen, *et al.* (1953). A thin window (2 mg./sq. cm.) geiger tube was used for radioactivity determinations.

Fatty Acid Experiments. Fatty acids were isolated from the alkaline hydrolysates of the intact rats reported upon in the previous communica-

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tion. Following the extraction of unsaponifiable matter the alkaline solution was acidified with concentrated hydrochloric acid to pH 1.0 and chilled overnight. The solid acids were skimmed off, and the solution was extracted exhaustively with pentane. The solid acids and the pentane-extracted acids were combined in pentane solution, washed with water, and the extracts evaporated under nitrogen. The crude fatty acids were assayed for radioactivity and then subjected to the lead soap fractionation of Twitchell (1919). The lead soaps of the solid fatty acids were recrystallized from ethyl alcohol-acetic acid, and regenerated with 10 per cent nitric acid. The regenerated solid fatty acids were then assayed; the liquid fatty acids were recovered from the lead soaps fractionation and assayed without further purification.

Radioactivity assay of the fatty acids involved plating the acids onto aluminum disks from a chloroform-pentane solution. Such samples weighed 1.80–2.20 mg., and were confined to a 2.0 sq. cm. area on the planchette. Duplicate analyses were made and the results corrected to a standard density thickness of 1.00 mg./sq. cm. by empirical self-absorption factors and then averaged.

Results

Table 1 presents the data collected for the radioactivity expired as CO_2 by PA deficient and non-deficient pair-fed rats. The specific activity and the Relative Isotope Content (RIC)[1] of the expired CO_2 from the PA deficient rats rose rapidly to a maximum within 10 minutes following acetate injection, while control rats attained specific activity maxima more slowly (within 20 minutes.) Both groups exhibited the same specific activities at their maxima, but the deficient animals maintained the high level for only 20 minutes while controls maintained their maxima for about 40 minutes.

The relationship between the two groups are more clearly presented in figure I where the RIC ratios (ratio of RIC for the PA deficient group to that of the control group) are plotted against the time following acetate injection. Here a 2.7-fold increase in RIC for the deficient animals over the RIC for the controls is attained 10 minutes after the administration of the tracer acetate. This early increase in RIC later drops to that of the controls.

[1] RIC is defined for these purposes as:

$$\text{RIC} = \frac{(\text{Counts/min./mg } BaCO_3) \times (\text{weight } BaCO_3\text{, mg.}) \times 10^5}{(6.0 \times 10^7 \text{ counts/min./100 g. of body weight}) \times (\text{body weight, in g.})}$$

This representation of yield or acetate conversion to CO_2 is proportional to the percentage yield or recovery of isotope, and considers the amount of CO_2 expired and the amount of radioactivity administered.

TABLE 1

Influence of PA Deficiency on the Rate of Expiration of Radioactive CO_2 in the Intact Rat

Time After Injection, min.	Specific Activity, Counts/min./mg. $BaCO_3$	Amount CO_2 Expired, mM.	Average CO_2 Expired/5 min., mM.	RIC
PA Deficient Group[1]				
0–5	257	2.71	2.71	1.03
5–10	1223	2.35	2.35	4.25
10–20	1133	2.67	1.34	4.40
20–30	1236	2.75	1.38	4.82
30–60	933	3.26	1.09	4.35
60–90	536	3.33	1.12	2.64
Pair-Fed Control Group[2]				
0–5	373	2.02	2.02	0.87
5–10	571	2.41	2.41	1.57
10–20	1008	2.21	1.11	2.39
20–30	1364	2.46	1.23	3.63
30–60	1184	3.00	1.00	3.89
60–90	695	3.44	1.15	2.63

[1] Average of 2 rats.
[2] Average of 3 rats.

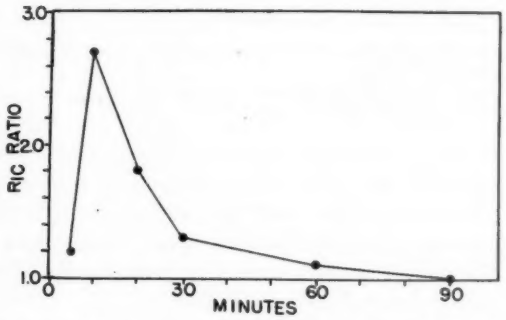


FIG. I. Ratio of RIC Factors for PA Deficient Rats to RIC Factors for Pair-fed Controls, CO_2 Excretion.

It appears that there is a linear decrease in specific activity from 30 minutes following injection of acetate in both control and PA deficient groups in the semi-logarithmic plot in figure II. The decrease in specific activity corresponds to an approximate half-life of 40 minutes, slightly higher than the 25 minute half-life reported by Gould, *et al.* (1949).

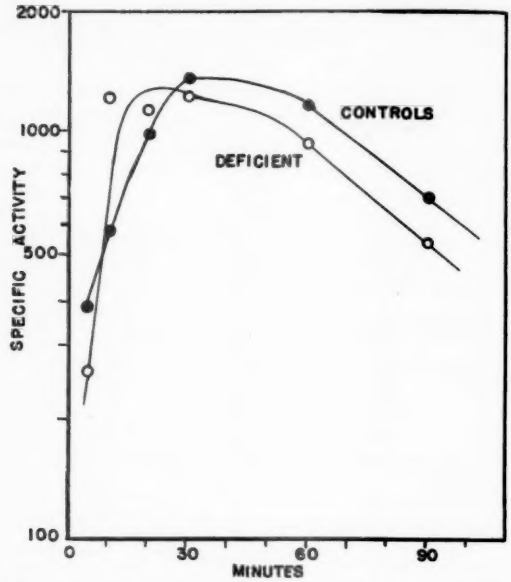


FIG. II. Semi-logarithmic Plot of the Specific Activity of $BaCO_3$ vs. Time Following Acetate Injection.
——o—— PA Deficient Group
——•—— Non-deficient Control Group

Because of experimental difficulties in the quantitative isolation of fatty acids the yields from the whole-rat hydrolysates are not believed to be reliable, and thus meaningful interpretations based on RIC or other measure of isotope incorporation are precluded. The trend involved in the changing specific activities of fatty acids is presented in figure III.

Discussion. In the intact animal the availability of tracer acetate for lipogenesis has generally been estimated from the amount of isotope appearing in the breath. By collecting samples of expired CO_2 at intervals following an i.p. injection of acetate-1-C^{14} information regarding the rate of metabolism, as well as the total availability of the labeled molecule, is obtained. Gould, *et al.* (1949) have proposed such a procedure for "application in the study of abnormal metabolic states in the intact animal." It is clear from the data of table 2 that on the basis of the total radio-

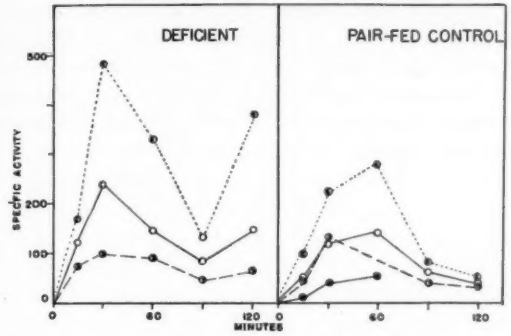


FIG. III. The Specific Activity of Fatty Acids Isolated from the Intact PA Deficient Rat.

——o—— Crude fatty acid fraction of deficient and *ad lib.*-fed non-deficient control groups, as noted.

- - -•- - - Saturated fatty acid fraction, deficient and *ad lib.*-fed non-deficient control groups, as noted.

— —o— — Unsaturated fatty acid fraction, deficient and *ad lib.*-fed non-deficient control groups, as noted.

——•—— Crude fatty acid fraction of pair-fed control group, non-deficient.

TABLE 2

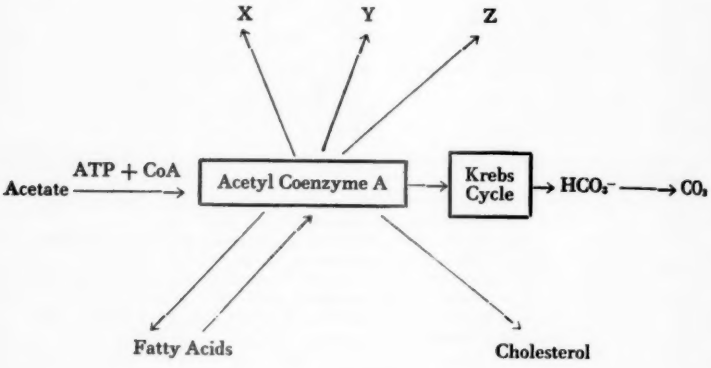
Specific Activities and Rates of Excretion of CO_2 Averaged Over the 90 Minutes of the Experiment

	Specific Activity, counts/min./mM. CO_2	Rate of Excretion, mM. CO_2/min./g. rat.
PA Deficient Group:	6.76×10^5	9.78×10^{-4}
Pair-fed Control Group:	6.85×10^5	6.09×10^{-4}

activity expired as CO_2 over a 90 minute period following acetate injection the acetate is equally available and equally metabolized in the deficient rats and pair-fed controls. The deficient animals, however, metabolize the acetate at a considerably higher average rate immediately following injection as well as attain a specific activity maximum earlier (Fig. II). The summation or averaging of results of this type may lead to erroneous conclusions and at best complicate the interpretations with respect to the more discrete metabolic stages.

The oxidation of acetate to CO_2 in mammalian systems is generally thought to proceed via acetyl coenzyme A and eventually through the Krebs cycle. The numerous competing systems

greatly complicate the picture (Fig. IV). Gould, *et al.* (1949) have shown that the rate of oxidation of acetate-1-C^{14} to CO_2 and the rate of CO_2 expiration are about the same in the "normal" intact rat. Detection of the minute differences in time involved, between enzymatic reaction and diffusion, etc., is not possible with the present data.



It would seem that a decrease in tissue coenzyme A induced by the PA deficiency would result in a decreased rate, and possibly decreased total in isotope incorporation into the various channels of figure IV. The reverse is actually the case as regards rate of incorporation of acetate into cholesterol and into oxidation products (CO_2), and possibly into fatty acids (Fig. III). In the case of the fatty acids only specific activities are considered; however, greater specific activities do prevail in fatty acids isolated from deficient animals. These differences are all the more striking when the fractionated fatty acids are examined. In that caloric restriction (pair-fed controls) results in a marked decrease in the incorporation of the label into the fatty acids (Fig. III) the elevated specific activities of fatty acids from the PA deficient animals is even more pronounced. No simple explanation of these findings is at hand, although certain aspects have been discussed in the first of these communications. This rapid attainment of maxima in specific activity is reminiscent of the rapidly metabolizing fatty acid fraction of mouse and rat liver (Hevesy, *et al.*,

1951; Hevesy, 1952; Ruyssen and Vandeputte-van Maele, 1951; Beeckmans and de Elliot, 1951).

The specific roles the steroid hormones play in metabolism are poorly understood, but it is known that a PA deficiency severely influences their syntheses, and conversely, that PA deficient animals are subject to more severe responses to stress than are non-deficient animals. Indeed the deficiency itself is a severe nutritional stress. It is not known what the relative influences were of the stresses of handling and injection on each animal. There were no gross differences in behavior; both PA deficient and non-deficient animals exhibited increased breathing rates and increased curiosity upon being placed in the respiration chamber, but all become placid within a few minutes. This situation is reflected in the increased amounts of CO_2 excreted during the first few minutes of the experiment.

Summary. Pantothenic acid deficient rats expire CO_2 following a single intraperitoneal injection of acetate-1-C^{14} slightly more rapidly than do pair-fed controls not PA deficient. Maxima in specific activity are attained within 5 to 10 minutes after injection in the deficient, within 10 to 20 minutes in control animals. Maxima in RIC are attained after 5 to 10 minutes in deficient rats and after 10 to 20 minutes in non-deficient animals. The total amount of isotope excreted as CO_2 over the first 90 minutes following injection is the same for both groups.

Maxima in specific activities of fatty acids of PA deficient and non-deficient rats are reached about 30 minutes following injection of acetate.

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THE INFLUENCE OF ALPHA TOCOPHEROL ON LUNG TUMORS IN STRAIN A MICE*

IRA R. TELFORD

The relation of vitamin E to tumor growth has been repeatedly investigated, frequently with contradictory results. Davidson (1935) and Cameron and Meltzer (1937) reported that hypervitaminosis E tends to retard tumor growth, but Okishio (1933), Severi (1934, 1935) claim that vitamin E favors the growth and/or incidence of tumors, while other workers maintain that the vitamin has no observable effect on tumor production (Engel, 1933; Marchesi, 1933; Zagami, 1933; Bryan and Mason, 1940). In view of these discrepancies we (Telford, 1949) reinvestigated the problem, substituting synthetic alpha tocopherol for the vitamin E concentrates used by the earlier workers. We reported that alpha tocopherol deficiency retards and hypervitaminosis E accelerates lung tumor growth in strain A mice. In this earlier work the mice were not held on isocaloric diets, which may have had some effect on the unequal tumor growth (Tannenbaum, 1940, 1942, 1947; and Larsen and Heston, 1945). Furthermore, the deficient animals had shown faulty absorption of the subcutaneously injected carcinogen, which may have accounted for the lower incidence and tumor count in the deficient group.

This paper reports an investigation using paired feeding of all animals, the utilization of technics which promoted uniform absorption of the carcinogen, and the use of a new method for the determination of tumor volume.

Materials and Methods

One hundred twenty-five young strain A mice of both sexes were studied. They were divided into three groups. Group 1 was held on a vitamin E deficient synthetic diet.[1] Group 2 was maintained on a normal commercial diet

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[1] Vitamin E deficient diet:

Casein	20.0	Salts (Hubbell's)	2.5
Corn starch	50.0	Brewer's yeast	7.5
Lard	18.0	Cod liver oil	2.0

(Purina Laboratory Chow). Group 3 received the deficient diet plus an oral supplement of 2 mg. synthetic alpha tocopherol[2] on alternate days.

To accelerate the appearance and growth of the experimental lung tumors, each animal received, at the beginning of the experiment, a subcutaneous injection of 1 mg. 1, 2, 5, 6, – dibenzanthracene in 0.25 cc. warm (40° C.) olive oil. By inserting a 1½ inch No. 24 hypodermic needle near the lower lumbar region, the carcinogen could be deposited subcutaneously in the dorsal scapular region of the animal. The latter area was then gently massaged for two minutes to spread the agent. This procedure resulted in uniform absorption of the carginogen in all groups with only an occasional small oil cyst appearing at the site of injection. Thus the difficulty of poor absorption of the carcinogen, which was characteristic of the deficient group in the earlier experiment, was largely eliminated.

Paired feedings were maintained in all groups so that the daily caloric intake of the normal and alpha tocopherol supplemented groups was equal to that of the deficient animals.

Five weeks after the injection of the carcinogen, 2 to 6 animals from each group were sacrificed at weekly intervals for 14 consecutive weeks. At necropsy the lungs were removed, fixed in 10 per cent formalin, and the number of external tumors counted. The left lung was then prepared for histological examination. Fifty μ serial sections, lightly stained with hemotoxylin, were prepared for an appraisal of both external and internal tumors. Projection drawings were made of each serial section and the tumors outlined. Using a compensating polar planimeter (Telford and Jeffery, 1953) the surface areas of the lungs and the tumors were determined and their respective total volumes calculated. Thus the conventional inaccurate method of counting only external tumors was avoided and replaced by a more satisfactory volumetric method.

Results and Discussion

Five weeks after the injection of the carcinogen, a 100 per cent incidence of lung tumors is noted in all groups regardless of diet or sex. The uniformly high incidence of tumors may have been influenced by the rather massive dose of the carcinogen used to accelerate their appearance. This observation nevertheless supports Heston's (1940) findings, and confirms our earlier impression (Telford, 1949) that the lower incidence of tumors in the deficient group may have been due to poor absorption of the carcinogen.

Seven weeks after injection of the carcinogen the autopsy findings show a definite sex trend in the number of external tumors per animal. The males have consistently more visible tumors than the females. This trend is followed in the subsequent weekly

[2] Generously supplied by Merck & Company, Rahway, N.J.

autopsies except in the eighth and sixteenth weeks, when the females have a slightly larger number of external tumors than the males. The average number of external tumors for all autopsies is 64.88 for males and 54.67 for females. Thus we confirmed our earlier findings that males in all experimental groups showed a somewhat larger number of external tumors per animal than the females, although these differences are not as great as previously stated (Telford, 1949). Lynch (1926) has also observed sex differences and reported 50 per cent of the male mice with pulmonary tumors as compared to only 25 per cent of the females.

A statistical analysis of the lung volumes shows no significant difference between the normal and deficient groups. By contrast the average lung size of the supplemented group is significantly greater than that of the other groups, the mean difference being 524 mm^3. Because of the greater lung size of the supplemented group, tumor volume as per cent of lung volume is not a valid transformation for analysis. There is no consistent lung volume trend in any of the three groups.

The significantly larger lung size in the supplemented animals might suggest that the alpha tocopherol may act as a growth stimulant. The final body weights of the supplemented animals, however, are not significantly different from either of the other groups. Therefore no general growth stimulation is present but differential organ growth may have occurred as in the case of the lungs. The same obscure mechanism may also account for the increased number of tumors in the supplemented group. Why these tumors show a strong tendency towards internal development is unknown.

From the statistical analysis of the data on tumor volumes there appears no significant difference between the normal and supplemented groups as to mean tumor volume or rate of development of the tumors. The deficient group, however, varies significantly from the normal and supplemented groups in that the mean tumor volume is smaller and the rate of development is about half as rapid as the other groups (Fig. I).

The pertinent information from the analysis of the tumor volumes is summarized in table 1. Since the normal and supplemented groups do not differ significantly either in mean volume or regression coefficient, the best comparison with the deficient

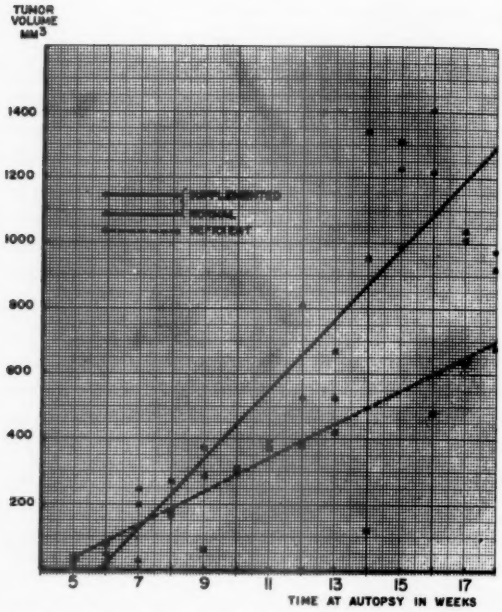

FIG. I. Showing lung tumor growth in Strain A mice held on alpha tocopherol deficient diet, normal diet, and alpha tocopherol supplemented diet.

group is based on the average of these two groups. These differences are displayed in figure I. It should not be assumed that the increase in tumor volume is necessarily a linear function of time. Variability among animals treated alike, as indicated by the mean square for error, is so great as to mask any exponential or logistic relationship which might exist in fact.

TABLE 1

Analysis of Tumor Volume Data

	n	Mean	"t"	Linear Regression Coefficient	"t"
1. Normal	26	539.0	....	105.4	9.911*
2. Supplement	58	590.6	....	107.8	34.546*
3. Deficient	41	383.1	....	50.9	4.891*
4. Mean of 1 and 2..	84	573.8	....	106.8	13.487*
5. 2–1	..	51.6	0.283	2.4	0.146
6. 4–3	..	190.7	4.093*	55.9	4.962*

* P less than 0.01.
Mean square for error 71,313 df = 78

Obviously, counts of visible tumors on gross lung specimens represent only a part of the total number of tumors. Internal tumors can best be accurately counted and measured on serial sections of the lung. The results of such a study on the left lung are summarized in table 2. The frequency of occurrence of tumors,

TABLE 2

Frequency of Occurrence of Lung Tumors in Mice

Treatment	n	Weighted Mean Frequencies		
		External Tumors	Internal Tumors	All
normal	26	46.8	25.3	72.1
α tocopherol supplement	58	56.6	62.1	118.7
α tocopherol deficient	41	50.7	24.9	75.6
All	125	52.6	42.3	94.9

and their relative distribution as to internal or external, do not differ significantly in normal and tocopherol deficient animals ($x^2 = 2.047$ and 2.613 respectively). Tumors occur with significantly greater frequency in supplemented animals ($x^2 = 647.923$) and show a strong tendency towards internal development ($x^2 = 400.215$) (Table 2), a fact that is particularly pertinent since internal tumors have been usually ignored in other lung tumor studies.

Tumor counts alone are of questionable value unless the volume of the tumor masses is determined. The data from this experiment emphasize that even though the normal animals show significantly fewer tumors than the supplemented group, the groups have no significant difference in lung tissue involvement. Therefore if the tumor counts alone are considered, as is usually the case, then it would appear that we substantiated our earlier findings (Telford, 1949) that hypervitaminosis E causes the animals actually more lung embarrassment than the normal diet; yet from the tumor volume data, it is obvious that neither the supplemented animals nor the normal group enjoy any significant difference in amount of protection from tumor growth. The tocopherol deficient mice, however, do have significant protection.

Conclusions. Neither hypo- or hypervitaminosis E has any effect upon the incidence of lung tumors in strain A mice five weeks after subcutaneous injection of 0.5 gm. of 1, 2, 5, 6 - diben-

zanthracene. The males show a greater number of external lung tumors than the females.

Alpha tocopheral supplemented mice have a significantly greater number of lung tumors than either the normal or deficient animals. There is no significant difference in the total tumor volume or rate of tumor development in either the normal or supplemented groups.

The tocopherol deficient group shows significantly smaller mean tumor volume, and slower rate of tumor development than the other groups.

The use of a compensating polar planimeter on serial lung sections furnishes an accurate volumetric method for the evaluation of tumor growth.

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SIMPLE NUTRIENT SOLUTIONS FOR ANIMAL CELLS*

CHARITY WAYMOUTH

Many properties and functions of the living cells of multicellular organisms can best be studied when these cells are isolated from the complexity of their natural environment. The physiologist has long been accustomed to studying surviving tissues and organs; the biochemist makes many of his experiments with tissue slices or breis. Typical biochemical or physiological experiments are of relatively short duration, extending over minutes or hours rather than days. Recently the virologist has come increasingly to use growing or surviving tissues or cells as vehicles for virus propagation and as test materials for virus assays. These may require survival or growth of the host cells for at least days, sometimes weeks.

At present, unlimited functioning and survival of metazoan cells *in vitro*, with or without proliferation, is possible only in media containing such biological variables as serum, tissue extracts or ascitic fluid. These materials introduce imprecisions into the system, reduce the reproducibility of experiments made with them and increase the difficulty of interpreting results. The need for adequate nutrient solutions of precisely known and reproducible composition has been felt from the time the Lewises (1911, 1912) initiated the search for them. Subsequent progress in analysis and synthesis of nutrient media for animal cells has been outlined elsewhere (Waymouth, 1954b). The form of artificial environment which has usually been provided for surviving cells is the so-called "physiological salt solution." There are many of these, including the still widely used solutions of Ringer (1886), Locke (1895, 1901), Tyrode (1910), Krebs and Henseleit (1932) and numerous variations upon them. The multiplicity of these solutions is evidence that no one of them has been found universally applicable.

Many "physiological solutions" have been designed to imitate serum in ionic composition, on the assumption that serum is a

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"natural," and therefore perhaps ideal, cellular environment. This assumption is apt to imply two misleading ideas. First, it suggests that a single nutrient medium (or, specifically, a single ionic environment) can be expected to be optimal for every type of cell. But different cells have different requirements. Distinctly different ionic requirements have been demonstrated for human adult epithelium and fibroblasts (Parshley and Simms, 1946, 1950); Pikovski (1954) has grown mammary gland tumor tissue in a medium of pH 9.3, a degree of alkalinity which damaged or completely destroyed normal mammary gland or connective tissue; Wolff, *et al.* (1953a) have shown that different organs, cultivated in synthetic media, have different vitamin and amino acid requirements. Moreover, conditions which are suitable for one cell type to carry out one metabolic function most efficiently are not necessarily the same conditions in which the same cell will perform another function optimally. Cohen (1945), writing on suspending media for metabolic experiments, discussed some aspects of this question. Two kinds of artificial environment should therefore be distinguished: one, in which a given cell can survive and act in a way as near as possible to its normal mode of operation *in vivo;* the other, in which a particular function is carried out at an optimum or maximum rate. The first kind of environment is truly "physiological," but will differ from cell type to cell type; the second kind of environment may be completely "unphysiological" in this sense. So Krebs (1950), though he presented a "serum substitute" based closely on the ionic composition of human serum, also designed other solutions, for special purposes, varying widely from this pattern, *e.g.*, his Medium II containing twenty times the amount of phosphate and one-tenth the bicarbonate required for a "physiological" solution.

The use of ionic serum substitutes may also lead to a second misconception, namely that salts alone can provide an innocuous milieu for cells. The power of cells to tolerate media with a wide range of ionic composition is very great. This is especially true in the presence of components of high molecular weight, such as the serum proteins, which exert a kind of "physiological cushioning" effect. But the capacity of cells for adjustment becomes progressively less as these complex components are diminished or excluded (*cf.* Gey and Gey, 1936; Richardson, 1946). The sim-

pler the composition of a physiological solution, the more attention must be paid to precisely defining the proportions and concentrations of the ingredients. This applies to the non-ionic as well as to the ionic part of the extracellular environment. The more searchingly we attempt to understand and define conditions for maintaining physiological equilibrium, the more profound becomes our disagreement with the statement of Stewart and Kirk (1954) that: "The place of physiological salt solutions in tissue cultures appears to be a relatively simple matter, and their functions are reasonably well understood." It would be truer to say that these functions have hitherto been masked, because salt solutions have been used only as minor components in complex biological media. Something better than a "physiological" salt solution is needed as a medium for maintaining a state of cell survival and function approximating the truly physiological.

The first two wholly defined nutrient solutions to go far beyond the older physiological solutions in their capacity to permit protracted cell survival were those described by White (1946, 1949) and by Morgan, Morton and Parker (1950). These contain, respectively, 25, 29, and 55 components in addition to inorganic salts, and permit survival of chick embryo tissues for average periods of 4 to 5 weeks.

The solutions to be discussed in this paper are believed to have advantages over the "physiological" salt solutions, in that they are nutritionally more adequate and permit prolonged survival of cells; over serum or other biological media, in that they are precisely defined; and over current defined solutions in their relative simplicity. In the design of these solutions consideration has been given to chemical stability. Components which are unstable on storage at 0° C. and at pH 7.4 to 7.8 have been omitted where possible.

Some substances are still included in the solutions, the individual needs for which have not been demonstrated. It is extremely probable that some of these will in future be omitted without impairing the usefulness of the solutions. Survival will undoubtedly be lengthened by addition of substances not now included. For practical purposes these details are not of immediate importance.

Materials and Methods

(a) *Cells.* In the early stages of the work, freshly explanted tissues of chick embryo heart (8 to 11 days) and lung (10 to 12 days) were the principal test objects. Cultures were made in roller tubes of both Willmer (1942) and Gey (1933) types. Tissue was attached to the tube wall with a minimum amount of a clot formed from human fibrinogen (0.2 to 0.3 per cent) and human thrombin (25 units per ml.). Later, following White (1946), the use of the clot was discontinued and the tissues allowed to grow directly on the glass of the culture tube. One ml. of the solution to be tested was added to each tube.

The later and more definitive experiments with chick embryo tissue (10 or 11 day embryonic heart and lung) were made in Gey roller tubes containing six fragments of tissue per tube. After an initial period of 2 to 4 hours to allow the tissue to adhere to the glass, 1 ml. of the test medium was put into each tube. The cultures were rotated at 15 revolutions per hour in an incubator at 38 ± 1° C. Fresh medium was supplied at 7 to 10 day intervals throughout the incubation period, which might be up to or over 100 days. Heart and lung were chosen mainly because each of these tissues exhibits in culture a form of visible activity which is a useful and immediately obvious criterion of persistent cell function (*cf.* White, 1946). Contraction of heart muscle can persist for long periods *in vitro*. After a few days in culture, a ciliated epithelium develops in cultures of chick lung, and the ciliary movements are readily seen. Even after these activitities have stopped, cells may be living, and their viability can be tested by observing whether or not migratory or proliferative activity reappears in the culture when a suitable biological nutrient is added.

Experiments have also been carried out with the strain L mouse connective tissue cells of Earle (Earle, 1943; Sanford, Earle, and Likely, 1948). In our hands, these cells have thrived better in stationary cultures (Carrel D3.5 flasks) than in roller tubes in a rotor turning at the conventional rate of 15 r.p.h. Stock cultures of strain L cells were at first kept in a medium (40 per cent horse serum, 20 per cent chick embryo extract, 40 per cent balanced salt solution) differing from Earle's standard nutrient only by a slight change in the composition of the salt solution. Later, because this medium maintained too high a rate of proliferation for our needs, the stock cultures were kept in a medium of 20 per cent horse serum, 10 per cent chick embryo extract and 70 per cent balanced salt solution. A simplified method for the preparation of these media has been described (Waymouth and White, 1954). Cultures for testing the chemically defined solutions were made from these stock cultures. One ml. of the test medium was put into each D3.5 Carrel flask, followed by about 0.3 ml. of a suspension of cells in stock medium (300,000 to 500,000 cells). In this way the cells were not subjected to the shock of an abrupt change of environment, as would be the case if a washed cell suspension were used as inoculum. After two days, and thereafter thrice weekly, the supernatant medium was discarded and renewed with 1 ml. of fresh test solution.

(b) *Nutrient solutions.* The medium which was taken as a starting point

for this series of experiments was that used by Wilson, Jackson, and Brues (1942), which is itself a modification of a medium of Baker (1936). The media of Baker, in turn, owed much to the preliminary studies of Vogelaar and Erlichman (1933). Wilson, Jackson, and Brues' medium, which contained Witte's peptone, a chemically undefined component, was first modified by replacing the peptone with a mixture of amino acids based closely on those used by White (1946, 1949). The large number of subsequent modifications—additions, subtractions and variations in the proportions of the components—will not be detailed here.

Each medium was prepared from a series of stock solutions (Table 1). Many variations have been made in the proportions of each stock solution in the complete medium. The most significant differences from the original solution of Wilson, Jackson, and Brues, and from the chemically defined media of White (1946, 1949), and of Parker (Morgan, Morton, and Parker, 1950; Healy, Fisher, and Parker, 1954) are in the amounts of phosphate and bicarbonate (Waymouth, 1954a, b). The amino acid mixture now differs from that of White (1949) in the proportions of the amino acids and in the inclusion of glutamine and tyrosine. One purine (hypoxanthine) is included. Among the substances tested and rejected as not essential for the fulfilment of the present objective are: ascorbic acid, cysteine, glutathione (all present in the medium of Wilson, *et al.*), inositol, p-aminobenzoic acid, β-carotene, α-tocopherol, vitamin D, vitamin B_{12}, adenine, guanine, thymine, uracil, sodium-β-glycerophosphate.

Each stock solution was made up in glass-distilled water and sterilized by passage through a No. 03 Selas porcelain bacteriological filter (maximum pore diameter, 1.2μ) (for details of filtration, see White, 1954). The final nutrient solutions were made up by mixing the components asceptically in the order and amounts shown in table 2. The compositions of the three solutions so obtained are given in table 3.

Commercial chemicals have been used throughout without further purification. It is recognized that this, particularly the use of commercial amino acids, may be a source of some variability and the means of introducing traces of unknown substances.

Results

Cultures of 10-day chick embryo heart remained healthy for 23 days in Wilson, Jackson, and Brues' (1942) medium with acid-hydrolysed Witte's peptone plus added tryptophan (10 mg. per 100 ml.; final nitrogen concentration, 6.9 mg. per 100 ml.). When White's (1949) amino acid mixture (nitrogen concentration 22 mg. per 100 ml.) was substituted for the hydrolysed peptone, the survival averaged 24 days with a maximum of 34 days. Addition of glutamine and hypoxanthine increased this maximum to 54 days. A still greater improvement in survival and promotion of mitotic activity was achieved by increasing the

TABLE 1

Stock Solutions

Stock Solution	Materials	mg./100 ml.
S/S 1.	NaCl	7000
	KCl	200
	$Ca(NO_3)_2.4H_2O$	260
	$MgCl_2.6H_2O$	100
S/S 2.	Na_2HPO_4	575
	KH_2PO_4	250
S/S 3.	$NaHCO_3$	5600
S/S 4.	Dextrose	10000
S/S 5.	Glycine	200
	L-lysine HCl	160
	L-methionine	50
	L-threonine	75
	DL-valine	125
	L-arginine HCl	75
	L-histidine HCl	75
	L-proline	50
	DL-isoleucine	50
	β-phenyl-L-alanine	50
	L-leucine	50
	L-tryptophan	40
	L-glutamic acid	150
	L-aspartic acid	60
	L-cystine	15
	L-tyrosine	40
	Phenol red, 0.5 ml. of 1.0 per cent solution	
	NaOH to pH 7.4	
S/S 6.	Thiamin HCl	20
	Calcium pantothenate	2
	Riboflavin	2
	Pyridoxin HCl	2
	Folic acid	0.4
	Biotin	0.4
S/S 7.	Hypoxanthine	50
	Glutamine	100
	Phenol red, 1 ml. of 1.0 per cent solution	

TABLE 2

Nutrient Solutions A, B, and C

	H_2O	S/S 1	S/S 2	S/S 3	S/S 4	S/S 5	S/S 6	S/S 7
Solution:								
A	64.5	10.0	4.0	4.0	5.0	5.0	2.5	5.0
B	61.5	10.0	3.0	3.0	5.0	10.0	2.5	5.0
C	62.0	7.5	4.0	4.0	5.0	10.0	2.5	5.0

phosphate to 6 times and the bicarbonate to 2.2 times the amounts in Wilson, Jackson and Brues' medium, *i.e.* to 2.35 mM phosphate and 26.6 mM bicarbonate respectively. It was found that

TABLE 3

Nutrient Solutions A, B, and C for Use With Chick Embryo Heart (Solution A); Lung (Solution B); and Strain L Mouse Cells (Solution C)

	mg./100 ml.		
	A (50/2)	B (48/2)	C (47/2)
NaCl	700	700	525
KCl	20	20	15
$Ca(NO_3)_2.4H_2O$	26	26	19.5
$MgCl_2.6H_2O$	10	10	7.5
Na_2HPO_4	23	17.25	23
KH_2PO_4	10	7.5	10
$NaHCO_3$	224	168	224
Dextrose	500	500	500
Glycine	10	20	20
L-lysine HCl	8	16	16
L-methionine	2.5	5	5
L-threonine	3.75	7.5	7.5
DL-valine	5	10	10
L-arginine HCl	3.75	7.5	7.5
L-histidine HCl	3.75	7.5	7.5
L-proline	2.5	5	5
DL-isoleucine	2.5	5	5
β-phenyl-L-alanine	2.5	5	5
L-leucine	2.5	5	5
L-tryptophan	2	4	4
L-glutamic acid	7.5	15	15
L-aspartic acid	3	6	6
L-cystine	0.75	1.5	1.5
L-tyrosine	2	4	4
Thiamin HCl	0.5	0.5	0.5
Calcium pantothenate	0.05	0.05	0.05
Riboflavin	0.05	0.05	0.05
Pyridoxin HCl	0.05	0.05	0.05
Folic acid	0.01	0.01	0.01
Biotin	0.01	0.01	0.01
Hypoxanthine	2.5	2.5	2.5
Glutamine	5	5	5
Phenol red	0.75	1	1

increasing the buffer salts to 2.35 mM phosphate and 26.6 mM bicarbonate required a corresponding increase in sugar concentration. There is, in solutions A and C, nearly a mole-to-mole ratio of dextrose to bicarbonate, and as an approximate rule this proportion, and the corresponding proportion of phosphates, should be maintained. Thus, the total concentration of buffer

can be again doubled, if the dextrose concentration is also doubled, but this confers no advantage and does not increase the survival time. Halving both of these concentrations results in a better solution than one in which either is reduced independently of the other, but survival and mitotic activity are reduced by comparison with solution A. For lung, the concentration of dextrose in solution B has been kept at 27.7 mM, with a buffer concentration 75 per cent of that in solutions A and C. By comparison of the survival times and general appearance of cells in nutrients (of which A, B, and C are examples) containing the seven stock solutions in various proportions, the conclusion was reached that each of the nutrient solutions, as given, had certain advantages for one of the three cell types tested.

Chick heart cultures in roller tubes remained in very good condition with persistent heart beat for about 40 days in nutrient solution A. Mitoses were common up to 30 days, occasional up to 90 days. Exceptionally, contractions of heart muscle continued to about 100 days. Fibroblasts survived in these cultures often for about 100 days, sometimes for up to 125 days.

Solution B supported the cells of chick lung for about 40 days, with ciliary activity evident between about the 6th and 30th days of culture. Mitoses in these cultures were rarely seen beyond the second week.

Cells transferred from a complete biological medium directly to a fully synthetic medium may suffer severe shock and damage with repression of growth and reduction of survival. Conversely, cells which have been successfully maintained in a synthetic medium for a prolonged period may at once disintegrate when exposed to a rich biological medium. The gradual adaptation to the environment was strongly stressed by Sanford, Earle, and Likely (1948) as an important factor in the survival and growth of single cells and the successful establishment of the single cell line of strain L. This factor was, however, neglected by the same workers (Evans, Shannon, Bryant, Waltz, Earle, and Sanford, 1953) in their study of the effects of synthetic media on the same strain of cells. Parker, Healy, and Fisher (1954) discussed some of the difficulties experienced in handling strain L cells in synthetic, as contrasted with biological, media. Strain L cells in our hands, even when carefully adapted to the nutrient solution C,

rapidly altered in morphology, losing their compact polyhedral appearance, becoming stellate, and developing very long terminal processes. Nevertheless, these cells, remained in moderately good condition for at least three weeks and survived more than 30 days in flask cultures in solution C, renewed thrice weekly. Active growth of cells which had been in this nutrient for 33 days was resumed when the cells were gradually readapted to a biological medium.

Discussion. Cells from embryonic chick heart and lung, and the strain L mouse connective tissue cells, have been maintained in direct contact with a glass surface and in fully defined nutrient solutions, without the addition of any complex biological materials. A slightly different solution has been found best for each type of cell. The solutions contain 25 components: dextrose, 17 amino acids, 6 vitamins of the B group and the purine hypoxanthine, in addition to inorganic salts. The proportions of the components vary. All three solutions are simpler than those of White (1949) which contained 29 constituents; of Morgan, Morton, and Parker (1950) with 55; and of Healy, Fisher, and Parker (1954) with 50. Yet the solutions A, B, and C have permitted regular survival of embryonic chick heart cells for over 100 days, of embryonic chick lung for 40 days, and of strain L mouse cells for over 30 days respectively. The chick cells were grown in roller tubes with nutrient renewals at 7 to 10 day intervals; the mouse cells were grown in Carrel flasks with change of the nutrient fluid three times a week.

It will be observed that the phosphate and bicarbonate contents of solutions A and C are high (2.35 mM and 26.6 mM respectively), compared with those of some of the common "physiological" solutions. The solution of Gey and Gey (1936) contains 1.0 mM and 3.0 mM, that of Earle (1943) 0.9 mM and 26.2 mM, and that of Hanks (1948) 0.86 or 1.44 mM, and 4.5 mM phosphate and bicarbonate respectively. The concentrations employed by White (1949) are 0.59 mM and 6.55 mM. As has been noted elsewhere (Waymouth, 1954a, b), inorganic phosphate represents only a small proportion, of the order of 25 per cent, of the total phosphate of serum. Chick embryonic extracts are also rich in organic phosphates (Davidson and Waymouth, 1946). The breakdown of organic phosphates, in media which contain them,

appears to make inorganic phosphorus available to the cells (Hanks, 1949; Harris, 1952). Therefore, nutrients from which biological sources of phosphate are excluded may be expected to require additional phosphate, either in organic or inorganic form, to that supplied in the common inorganic diluting solutions. We have at present no evidence that any organic source of phosphate confers advantage over inorganic salts in maintenance media such as solutions A, B, and C. Similarly, almost all the older "physiological salines" contained amounts of bicarbonate lower than in mammalian serum (Waymouth, 1954b). Exceptions are Earle's (1943) saline containing 26.2 mM, and Krebs' (1950) "serum substitute" containing 25 mM. Harris (1954) demonstrated that bicarbonate was essential for chick fibroblast outgrowth, which was doubled by increasing the concentration of $NaHCO_3$ from 100 to 200 mg. per 100 ml. (11.9 to 23.8 mM). Parker's nutrients Nos. 199 and 703 contain the salts of Earle's saline. They are therefore satisfactory in bicarbonate but somewhat deficient (1.0 mM) in phosphate. They are also low in dextrose (5.5 mM), compared with solutions A and C (27.7 mM).

The nutrient solutions here described are believed to possess practical advantages as suspending media for living cells in a variety of experimental systems. They are relatively stable, simple, reproducible solutions, capable of providing well-defined conditions for experiments requiring long- or short-term survival of cells. In an unsupplemented balance salt-dextrose solution, survival may vary from a few hours for leucocytes of the myeloid series to about 10 days for some connective tissue cells. Many experiments demand longer survival than this. It has been shown that the addition to such a "physiological" solution of a few defined components, namely some of the B vitamins which act as cofactors in important metabolic processes, some amino acids and hypoxanthine, will greatly prolong cell life and function, including a limited amount of proliferation. This gives greater assurance that, even early in an experimental period, the functions of the cells are essentially unimpaired.

The work of Wolff and his collaborators has attested to the value of similar simple supplements to physiological solutions for the development *in vitro* of embryonic organs of the chick

and duck (Haffen and Wolff, 1953; Wolff and Haffen, 1953; Et. Wolff, 1953; Wolff, Haffen, Kieny, and Wolff, 1953a, b). These studies also demonstrated that survival and differentiation depended upon different supplements from those necessary for proliferation, and that the requirements of different organs (gonads, tibiae, syringes) for these functions were different (Em. Wolff, 1953; Haffen, 1953; Wolff *et al.*, 1953a, b; Kieny,1953).

Physiological maintenance media of the type recommended here may prove useful to virologists. These fully defined solutions are capable of supporting a degree of normal function comparable to that achieved in the more costly and complex solutions of White and Parker. Actively metabolizing and growing cells are necessary for the multiplication of some viruses. Others will multiply in slowly growing cells (Scherer, 1953). The theoretical advantages of protein-free suspending fluids for virus studies are well known (*e.g.* Melnick and Riordan, 1952; Pereira, 1953). One of the solutions described here (solution C = CW47/2) has already been extensively tested by Hotchin (1954). He obtained much more uniform layers of chick and monkey cells for virus plaque assay in solution C than with the usual medium (10 per cent chick embryo extract and 20 per cent horse serum in Earle's saline). Western equine encephalomyelitis virus multiplied in chick embryo tissue in solution C, giving higher titres than in any other medium tried. Poliomyelitis virus grew better in monkey kidney cells in solution C than in the usual medium (10 per cent embryo extract in Earle's saline), and the growth equalled that in the more complex synthetic medium No. 199 of Parker. The viruses of fowl plague and of the common cold were also cultivated in tissues in solution C, with results as good as those in any other medium. All the virus experiments of Hotchin were of 3 to 10 days duration.

For biochemical studies, the potential value of the type of environment devised for tissue cultures has been pointed out by Stewart and Kirk (1954). Their review of liquid media used in tissue culture however, deals exclusively with biological, as contrasted with chemically defined, nutrients. It is hoped that precisely defined nutrient solutions like those presented in this paper may be useful in providing conditions approximating the physiological, where these, rather than conditions "optimum" or

"maximum" for short term study of particular metabolic reactions are required. The latter type of experiment may call for highly unphysiological solutions, such as the medium of Le Page (1948), which is sodium-free and has a tonicity 65 per cent of that of an "isotonic saline" and is designed to be optimum for sustaining phosphate bond energy.

The nutrient solutions A, B, and C can undoubtedly be improved upon. They are made available now in their present form because they have already proved useful and in the hope that their usefulness may be extended.

Summary. Three chemically defined nutrient solutions are described, containing salts, dextrose, amino acids, hypoxanthine and six of the B vitamins. The life and function, with some proliferation, of chick and mouse cells have been maintained in these solutions for far longer than is possible in "physiological saline solutions." The solutions are presented as potentially useful for biochemical, physiological and virological work with surviving cells. They have the advantage over serum or other biological media of being reproducible. They are also stable and are less elaborate than the synthetic media hitherto available.

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A STATISTICAL ANALYSIS OF THE GROWTH IN LENGTH OF THE FILARIAL WORMS IN THE COTTON RAT*

MARY G. WESTBROOK AND J. ALLEN SCOTT

In experiments on immunity to the filarial worms, *Litomosoides carinii*, the rate of growth in length has proved to be an important criterion. For example, Macdonald and Scott (1953) found that the worms grow more slowly in cotton rats which have been previously infected than in cotton rats which have received no previous infection. Furthermore Scott (1947) observed a slower rate of growth of the worms in the white rats even when this species had not received a previous infection. Both of these conclusions were based on comparisons of the average length of worms of the same age from the different groups of hosts under consideration. It seemed likely that study of the entire growth curve of the worms in non-immune cotton rats would provide a basis against which growth in immune cotton rats or in other species of rodents could be compared. By such comparisons it might be possible to select critical periods in the growth cycle and to design experiments at these periods which would aid in elucidating the mechanism of both acquired and racial immunity. This paper presents a statistical analysis of the average growth in length of filarial worms of known age in non-immune cotton rats.

In natural infections the adult worms are located in the pleural cavity of the cotton rat. The adult female worms produce precocious embryos known as microfilariae which penetrate into the circulatory system of the host and can be found circulating in the peripheral blood of the host. When the tropical rat mite, *Bdellonyssus bacoti*, bites the cotton rat, it ingests these microfilariae with its blood meal. In the mite the microfilariae develop into larvae which increase in size and undergo several molts until the so-called third or infective stage is reached. At this

*Contribution from the Department of Preventive Medicine and Public Health and the Laboratory of Helminth Research, The University of Texas Medical Branch, Galveston, condensed from a thesis presented by Miss Westbrook in partial fulfillment of the requirements for the degree of Master of Arts.

stage, growth and development are arrested in the mite. When the mite again bites a cotton rat, the infective larvae enter the skin of the rat and migrate to the pleural cavity within a period of a few days and there resume their growth and development. Approximately 8 to 10 days after introduction into the rat they molt into the fourth larval stage in which they continue to grow and develop. Soon after the 20th day, they molt into the adult stage during which their growth is completed. Further details regarding the time of molting will be presented later. The morphology of these various stages has been described by Cross and Scott (1947) and Scott, *et al.* (1951).

Methods and Materials. The cotton rats used in this study were laboratory-born rats of a strain derived from wild *Sigmodon hispidus texianus* caught in Galveston County, Texas. These cotton rats were not infected for the purpose of this growth study, but served as control animals for various experiments performed in this laboratory in the past several years. The worms used for infection were obtained from strains originally harbored by these same wild cotton rats. The mites in which the infective larvae developed were from a strain originally isolated from the same wild rats.

The cotton rats were experimentally infected by the method described by Scott and Macdonald (1953). The mites were dissected in half-strength Tyrode's solution and the larvae removed. These larvae were then examined to eliminate any which had been damaged or which had not attained the morphological characteristics of the infective stage. The remaining infective larvae were counted before being introduced into a subcutaneous pocket formed by a surgical incision in the side of the rat. The incision was then sutured. At autopsy, the pleural cavity was opened and the large worms removed with a bent wire and placed in salt solution. Then the pleural cavity was repeatedly washed with salt solution to recover any small worms present. It has been shown that this method results in virtually 100 percent recovery of the worms in the pleural cavity. The worms were fixed in 70 percent alcohol at 70° C. Most of the worms more than 2 cm. in length were transferred to 70 percent alcohol containing 5 percent glycerin. The smaller worms were passed through graded glycerin-alcohol mixtures and mounted in glychrogel

(Cross and Scott, 1947). Enlarged images of the worms were projected on a paper screen along with a scale for calibration. Tracings of these images were measured with a map measuring device. The measured worms were examined microscopically to determine their sex and morphological stage of development as well as to eliminate any which were broken and incomplete.

Presentation of Data. Measurements were available on 753 male worms and 830 female worms from 48 cotton rats. Data concerning the measurements of the individual worms are available in the Laboratory of Helminth Research and summaries of these data for each individual rat are given in a thesis by Westbrook (1954). These summaries include the age, sex and weight of each cotton rat, the age of the larvae in the mite, and the number of larvae given in the infecting dose. the percentage of these larvae recovered alive at autopsy, the number of worms of each sex measured and the mean length of all worms of each sex from each rat, the latter called for convenience the "rat mean."

The data shown in table 1 were also derived from the same observations. The lengths of all worms of each sex, irrespective of their morphological stage of development or the rat from which they came, were added to obtain the "day means," *i.e.* the mean length of all worms of a given "age." The term "age" of the worms as used throughout this paper refers not to the actual age but to the number of days from infection of the rat to autopsy. The actual age would include a period we designate the "age of the microfilaria," *i.e.*, the time from parturition to ingestion by the mite, and a period we designate the "age" of the larvae in the mite, *i.e.*, the period from ingestion of the microfilaria to the introduction of the infective larvae into the rat. The age of the microfilaria cannot be determined but the average age is probably more or less constant, under the conditions of these experiments. The age of the larvae in the mite will be discussed below. The estimates of variance shown in this table will also be discussed below.

The Course of Growth. In studying the growth of a free living animal it is sometimes possible to make measurements on the same individual at successive intervals of time. Several similar animals living under similar environmental conditions can be measured in this manner and the average of their measurements

computed for each selected time interval. Considering the group of animals measured to be a random sample from a population of similar animals living under the same conditions, a curve fitted to these observed averages would represent an estimate of the population curve. The growth of a parasite, however, cannot be studied by this method for it is impossible to make successive measurements on the same individual parasite over a period of time. The influence of the host on the parasite's development is another uncontrollable factor in studying parasitic growth. It is therefore necessary to measure different individuals grown in different hosts for various periods of time and to use the averages of these measurements to estimate the population curve.

TABLE 1

Number and Mean Length of All Worms of Each Sex on Given Days After the Time of Infecti the Standard Errors of These Day Means and Other Estimates of Variance

			Male Worms				Female Worms				
Age of Worms	No. of Rats	No. of Worms	Estimates of Variance		Day Mean	Stand. Error	No. of Worms	Estimates of Variance		Day Mean	Stand Error
			Rat	Worm				Rat	Worm		
t	n	k	s^2_r	s^2_w	$\bar{X}$	$s_{\bar{x}}$	k	s^2_r	s^2_w	$\bar{X}$	$s_{\bar{x}}$
12	1	37	...	.05	1.20	.04	24	....	.05	1.34	.04
14	1	23	...	.17	1.70	.09	15	....	.18	1.80	.11
15	1	8	...	.14	2.61	.13	8	....	.84	3.10	.32
20	1	9	...	1.83	4.99	.45	4	....	1.32	6.85	.57
22	1	12	...	1.57	5.27	.36	19	....	5.41	7.40	.53
23	2	32	0.00	2.36	7.16	.86	25	0.00	.86	9.73	.18
24	27	478	1.67	2.10	7.40	.26	531	.14	2.85	9.27	.10
25	2	28	3.04	1.67	7.11	1.26	30	4.24	3.37	10.02	1.46
30	2	39	2.32	6.13	8.67	1.15	47	2.54	10.79	9.77	1.22
35	1	4	...	.25	15.25	.25	11	....	17.87	46.46	1.27
37	5	54	2.23	4.33	14.76	.73	74	48.92	70.66	37.82	3.26
38	2	18	1.65	.57	15.56	.92	20	6.86	11.36	45.10	2.00

In fitting a growth curve to any set of observations, assumptions must be made concerning the shape of the growth curve. The curve may be composed of a single cycle or of multiple cycles, but in either case, the shape of each cycle is sigmoid in form. A sigmoid curve may be either symmetrical or skewed, whether it represents the complete growth of an organism or one of several cycles of growth. Any sigmoid curve has two segments, the first having an increasing rate of increase, and the second a decreasing rate of increase. The point at which these two seg-

ments are joined is called the point of inflection. In a symmetrical curve the point of inflection is reached when the organism has attained half its ultimate size and the second segment, if reversed and inverted, would coincide with the first segment. In the case of the skew curve, the point of inflection is reached either before or after the organism has attained half of its ultimate size and the two segments are therefore not similar.

The available data are not sufficiently extensive to determine whether growth of these filarial worms in the cotton rat occurs in a single cycle or in several cycles. As in many invertebrate animals, the development of this species is characterized by metamorphosis. The course of growth of these worms differs, however, from such invertebrates as the arthropods. In the arthropod there is a sudden increase in size at the time of molting but very little increase occurs during each stage because of the presence of a rigid exoskeleton. In these worms most of the increase in size is attained within each morphological stage. While a relatively small increase occurs during the molt. It is possible that there is a separate cycle of growth present in each morphological stage. Evidence regarding this point will be presented later. The most satisfactory fit to these data was obtained, however, by the assumption that the growth of the worms in the rat follows a single cycle. It was impossible to determine whether a symmetrical or skewed curve would provide the best fit to this cycle since few observations were made near the upper asymptote. The symmetrical form was therefore adopted as a matter of convenience.

One of the principal points of interest in this study was analysis of the variability in the length of the worms at various ages. We hoped that such an analysis could be used in the form of confidence limits about the growth curve to test the significance of differences between the growth in immune hosts and that in non-immune hosts. Since consideration of such confidence limits in the mathematical literature on growth curves has been limited to the Pearl-Reed logistic, this type of curve was selected for application here.

Fitting the Logistic Curve. The general equation for the Pearl-Reed logistic curves used in this study is given by Reed and Berkson (1929) as follows:

$$y - d = \frac{K}{1 + C e^{rt}}$$

In this equation t is a specified "age" in days of a group of worms. Y is the "day mean" for the specified age, *i.e.*, it is the mean length of all the worms of that age irrespective of the stage of morphological development or the rat from which they came. C and r are constants to be discussed below. d is the lower asymptote and K is the distance between the upper and lower asymptotes. The method of fitting the logistic curve was a combination of the method of three equidistant points (Pearl, 1924; Spurr and Arnold, 1948) and the logarithmic-graphic method (Pearl, 1940). In both these methods, d and K must be estimated from the observed data.

By the use of a lower asymptote other than zero, it is implied that the previous growth of the larvae in the mite followed at least one separate growth cycle. This assumption was adopted because it produced a better fit than the assumption that the lower asymptote was equal to zero. The first attempt to estimate the value of d was based on the work of Scott, *et al.* (1951), who found that the average length of 41 third stage larvae was .85 mm. A better fit to these data was obtained, however, by the use of .83 mm. as the value of d.

The first estimate of K was made by the three equidistant point method as described by Spurr and Arnold (1948). The "best" estimate of the true K value is made when the first point falls near the lower asymptote. The second point is near the point of inflection, and the third point is near the upper asymptote. As three such widely separated equidistant points were not available, K was estimated from each of several sets of three points and these estimates were tested by the logarithmic graphic method. In this graphic method

$$Z = \frac{K - (y - d)}{(y - d)}$$

and log Z should plot as a straight line when K and d have been correctly estimated. The selection of the final K was made by inspection and the straight line was fitted graphically. The slope (m) of this straight line was then calculated. The rate constant of the logistic (r) was calculated by taking 2.30259 m while the

constant of integration (C) was found by determining the value of Z at t = 0. The equation for the male worms was found to be:

$$Y_c - .83 = \frac{15}{1 + 569.26\, e^{-.2541\, t}}$$

where Y_c is the calculated or theoretical value of the mean length on a specified day (t). The equation for the female worms was found to be:

$$Y_c - .83 = \frac{63}{1 + 858.15\, e^{-.2045\, t}}$$

The theoretical curves calculated from these two equations are shown, along with the observed day means, in figure I.

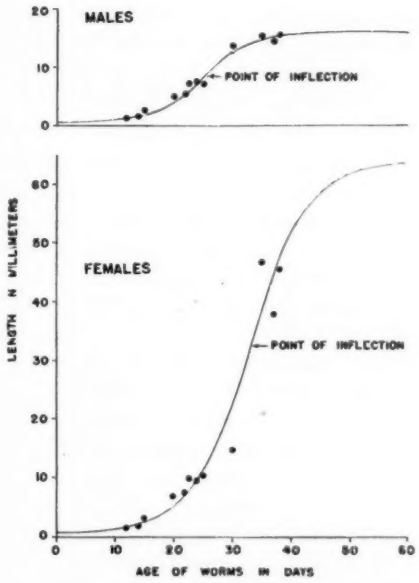

FIG. I. The logistic growth curves for male and female worms.

The most obvious difference between the growth curves of the two sexes is that the females attain a much larger ultimate size (K) than the males. A second difference is that the point of inflection occurs at 25 days in the curve for the males as compared

with 33 days in the curve for the females. In other words, the males attain half of their ultimate size at an earlier age than the females.

The values of K are not very well established because of the lack of observations near the upper asymptote. It is entirely possible that further measurements of older worms will show these K values to be too low. If so, it would be necessary to fit skew logistic curves (Pearl, 1924), the first half of which would essentially coincide with these symmetrical curves. On the other hand, the values of K estimated here are consistent with those obtained by Scott (1946) who made more observations near the upper asymptote. His data, however, were derived quite differently and no exact comparison is possible.

Tests For Goodness of Fit. It is clear from inspection of figure I that most of the points are fitted reasonably well. The means of female worms on days 30, 35 and 37, however, show an excessive amount of deviation. A careful study of the original data revealed no satisfactory explanation for this excessive deviation.

Since the parameters of the curves were derived in part by estimation, it seemed wise to apply tests for the goodness of fit. No satisfactory methods for this purpose are available. To test the fit of his curves Pearl (1940) used the "root mean square deviation" which is sometimes referred to as the "standard error of estimate." This error term is equal to the square root of the quotient formed by dividing the sum of the squared deviations of the observed values from the calculated points by the number of observed values. For the same purpose, Schultz (1930) used the "quadratic mean square error" which he calculated in the same manner as the root mean square deviation except that he reduced the number of observed values in the denominator of the fraction by the number of constants used in fitting the curve. For the curves presented here the root mean square deviation was 1.16 for male worms and 4.92 for female worms. The quadratic mean square error was 1.74 for the male worms and 7.38 for female worms. The male curve, therefore, appears to provide a better fit than the female curve since both error terms would equal zero if the curves were a perfect fit.

Neither the root mean square deviation nor the quadratic mean square error is, however, an adequate test of the goodness of fit.

The chi square test for goodness of fit was therefore applied even though some of the underlying mathematical assumptions of this test may not be fulfilled. The degrees of freedom were found by the usual rule of the number of observations minus the number of constants in the fitted curve, *i.e.*, d.f. $= 12 - 4 = 8$.

The male curve had a chi square of 1.954 ($P = .98$). The female curve had a chi square of 4.804 ($P = .68$). When the 30 day mean of the female worms was omitted, the P value increased from .68 to .80. Therefore, both curves can be considered a good fit to the observations, but the male curve is a much better fit than the female curve. This same conclusion was drawn from the root mean square deviation values and the quadratic mean square error terms, but the chi square test provides additional information concerning the probability of obtaining such curves by random sampling from the specified population functions.

Stage of Development. The previous discussion has been based on the mean length of all the worms of a given age and sex irrespective of their morphological stage of development, and has dealt with the hypothesis that growth occurs as a single cycle. Consideration should also be given to the alternate hypothesis that there is a separate growth cycle in each morphological stage. The present series of data includes only a few observations at the time of molting from the third to the fourth larval stage. Other data (Scott, *et al.*, 1951) show that the morphological changes which occur at this molt are similar to those occurring at the molt from the fourth to the adult stage. The differentiation of stages is based on the fact that the shape of the sclerotized stoma is characteristic for each stage. The definition of molting worms used here differs somewhat from that used by Macdonald and Scott (1953). In this paper molting is considered to begin with the first definite appearance of the new stoma surrounding the old stoma and to end with the complete detachment of the old cuticula and old stoma from the new ones even if the worm is still within the old cuticula.

An important factor in considering the course of growth is the variable time at which the worms reach the molting stage. The proportion of worms in the different stages or in the process of molting at various periods is shown in figures II and III. The earliest observations in this series were on the 12th day after

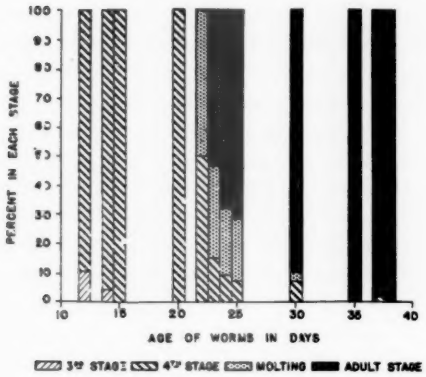

FIG. II. The percentage of male worms in each stage of development at various ages.

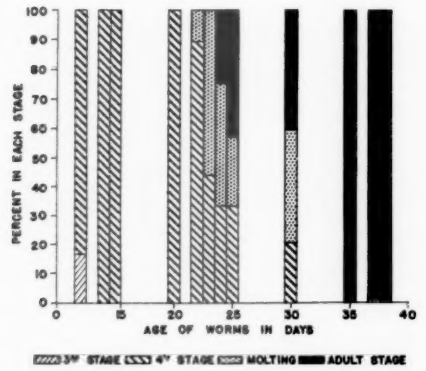

FIG. III. The percentage of female worms in each stage of development at various ages.

infection at which time most of the worms of both sexes were in the fourth larval stage. By the 22nd day half of the male worms had started to molt. On the next day all but about 15 percent had at least started to molt, while over half had already completed the molt and were in the adult stage. In the case of the female worms, about 10 percent had started to molt by the 22nd day, half had started by the 23rd day, but none completed the molt until the 24th day, at which time about one-fourth of the worms

were in the adult stage. Hence the males seem to be ahead of the females in their morphological development by at least one day. Some worms of either sex do not molt until considerably later than the average. If the time at which molting occurs were plotted as a frequency curve, it would apparently be definitely skewed to the right because of the delayed molting of some worms. The tendency of the 30-day worms to deviate markedly from the general pattern is again evident here.

Another aspect of the variable time of molting is evident in considering the mean length of worms in the different stages at various ages as shown in figures IV and V. The most interesting

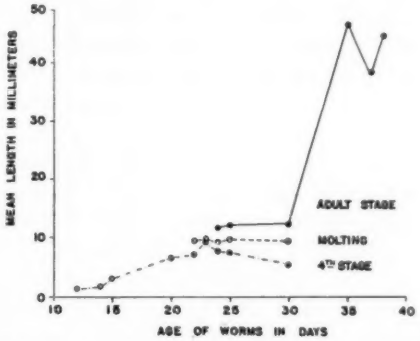

FIG. IV. The mean length of male worms by stage of development at various ages.

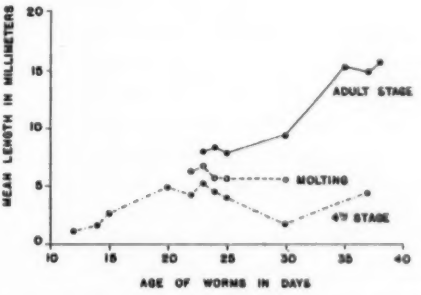

FIG. V. The mean length of female worms by stage of development at various ages.

and obvious conclusion from these data is that, on the average, molting seems to occur when a certain size has been attained. This conclusion arises from the fact that the mean length of the molting worms is relatively constant regardless of the age of the worms. Of course, there is considerable variability in the length of individual molting worms about this average length. It would seem, however, that there is greater variability in the rate of growth of the individual worms than in their size at the time of molting.

An attempt was made to fit growth curves separately to the fourth stage and to the adult stage, but the variability mentioned above was so great that it was virtually impossible to obtain a satisfactory fit with the amount of data now available. Until more data can be obtained it must be concluded that separate growth cycles in the different stages may exist, but the best fit can still be obtained with the assumption of a single cycle of growth.

Analysis of the Variability in Length of Worms. An ideal experiment for the study of the growth of these worms would have an orthogonal design, *i.e.*, there would be the same number of worms of each sex from each rat and the same number of rats on each day, or at least on each of a series of evenly spaced days. The analysis of variance could then be applied to determine the effect of the various elements of variability involved. Unfortunately it is impossible to perform such an ideal experiment. It would be possible, barring accidental deaths, to have the same number of rats on evenly spaced days. But even when rats are infected with the same number of larvae, both the total number of worms recovered and their sex ratio will be variable. Although the available data are far from ideal and a single analysis of variance solution is impossible, the same components of variance are present and some of them are susceptible to analysis.

The variability in length of the worms must of course be considered separately for each sex. For each sex the variability can be divided into the "within day" variability and the "between day" variability. The within day variability, *i.e.*, the variability in length of worms of a given age and sex, has two principal components. The first is the "within rat" variability, which is variability in length of the worms from a given rat about their mean,

i.e., about the "rat mean." This component is measured by the "worm variance." The second component is the "between rat" variability, which is the variability of the various rat means about the day mean and is measured by the "rat variance." The between day variability, *i.e.*, the variability between worms of different ages, also has several components. One of these components is the variability due to curvilinear regression, which is an expression of the fundamental growth process, namely, the increasing average length of the worms associated with increasing age. The remaining components of between day variability can be grouped together under the term "variability not due to regression." The latter portion of the variability is expressed by the deviations of the day means about the fitted curve, a point already discussed in connection with consideration of the goodness of fit. In addition to these various components which can be identified there must be numerous interactions, many of which would be associated with the fact that some components of both within day and between day variability show an increase in size proportional to the increasing length of the worms.

Although analysis of variance as a single over all solution which would isolate the components of between day variability was impossible, because of the unequal numbers of worms and rats, it was possible to apply analysis of variance to the data for each day separately. Table 2 shows the form of this analysis of

TABLE 2

Form of Analysis of Variance for Each Day

Source	Degrees of Freedom	Mean Square as Estimate of Variance
Rat (between rats)	n–1	$\sigma_w^2 + k_o \sigma_r^2$
Worms (within rats)	$\Sigma k - n$	σ_w^2
Total	$\Sigma k - 1$	

variance including estimates of the population variance based on rat mean square and on worm mean square. Because of the unequal number of worms per rat, the estimates based on the rat mean square involves the use of Snedecor's (1946) "average number of items, k_o" which in this application represents the

"average" number of worms per rat. If k equals the number of worms per rat and n equals the number of rats per day,

$$k_o = \frac{1}{n-1}\left(\Sigma k - \frac{\Sigma k^2}{\Sigma k}\right)$$

The worm mean square (s_w^2) based on a particular day's sample estimates the population worm variance (σ_w^2) for that day. The population rat variance (σ_r^2) for a particular day is estimated by the sample rat variance (s_r^2) for that day calculated as follows:

$$S_r^2 = \frac{\text{Rat mean square} - \text{Worm mean square}}{k_o}$$

The values of s_r^2 can only be calculated for those days represented by more than one rat, viz., days, 23, 24, 25, 30, 37 and 38. These values are shown in table 1 and were found to be significant at the 5 percent level on 5 of 6 days when tested by the F test with

$$F = \frac{\text{Rat mean square}}{\text{Worm mean square}}$$

On the 23rd day the worm mean square was larger than the rat mean square hence it was concluded that there was no rat effect on this day and s_r^2 was entered as zero. The sample worm variances (s_w^2) were also entered in table 1 for these 6 days. For the other days represented by only one rat s_w^2 was calculated by dividing the sum of the squared deviations about the day mean by the number of worms minus one. Table 1 also shows the standard errors of the day means which were calculated as follows:

$$S\frac{2}{x} = \frac{s_r^2}{\text{Number of rats}} + \frac{s_w^2}{\text{Number of worms}}$$

The conclusions which can be drawn from these estimates of population variance are principally that the worm variance tends to increase with the age of the worms while the rat variance does not show a similar tendency.

As another phase in the analysis of the variability in length of worms of the same age, the data regarding 24 day old worms

were broken down by some of the factors which might influence the growth of the worms. The 24 day old worms were selected for study because they are the largest sample of worms and came from the largest number of cotton rats. The factors to be considered are (1) sex of rat, (2) age of rat at time of infection, (3) weight of rat at death, (4) age of larvae in mites, (5) number of larvae given in the infecting dose, and (6) time of year of the experiment. Correlation tables were constructed to compare the mean lengths of the worms in the rats with each one of these factors except the sex of the rat. None of these comparisons showed a significant correlation.

The correlation table concerning the age of the rats showed that the rat means fell into two fairly distinct groups. The first group included means from rats between 6 months and one year of age, and the second group included means from rats over one year of age. These groups were designated as "young" and "old" respectively, and the comparisons made between the mean length of worms from each of these age groups and from the two sex groups are shown in table 3. The conclusions from these

TABLE 3

Comparisons of the Mean Length of 24 Day Old Worms by Age and Sex of Rats

Sex of Worms	Rat Comparisons X_1	X_2	N_1	$\bar{X}_1$	N_2	$\bar{X}_2$	t	P
Male Worms	Total Males vs. Total Females		244	7.83	234	6.94	5.23	<.001
	Young Males vs. Young Females		150	7.16	164	6.63	2.74	<.01
	Old Males vs. Old Females		94	8.91	70	7.68	4.44	<.001
	Young Males vs. Old Males		150	7.16	94	8.91	7.88	<.001
	Young Females vs. Old Females		164	6.63	70	7.68	4.16	<.001
Female Worms	Total Males vs. Total Females		262	10.07	269	8.77	6.67	<.001
	Young Males vs. Young Females		148	9.11	179	8.36	3.11	<.01
	Old Males vs. Old Females		114	11.32	90	9.58	6.49	<.001
	Young Males vs. Old Males		148	9.11	114	11.32	8.99	<.001
	Young Females vs. Old Females		179	8.36	90	9.58	4.34	<.001

comparisons were the same for both sexes of worms. On the average, worms from male rats of either age group were significantly larger than those from female rats of the same age group, and worms from old rats of either sex were significantly longer than those from young rats of the same sex.

Further comparisons between the age and sex groups of the 24th day were made on the basis of the mean length of the worms in each morphological stage and on the basis of the percentage in each group which had at least reached the molting stage. The previous conclusions were in general supported by these additional comparisons. The few exceptions observed were associated for the most part with small numbers of worms or with small and insignificant differences.

Similar detailed comparisons could not be made for the other days because of the small number of observations, but scrutiny of the data reveals no reason to believe that the conclusions would not hold true at all ages. The conclusions that worms grow more rapidly in old rats than in young rats and more rapidly in male rats than in female rats may eventually point the way to new experiments on the underlying factors. The immediate applications of these conclusions is principally that future experiments with the filarial worms involving comparisons of the length of worms in experimental and control cotton rats should be done with rats of the same age and sex.

Confidence Limits. The logistic curves which were fitted to the day means for male and female worms are estimates of the population curves. These population curves are thereby assumed to be logistic functions with an infinite number of worm lengths normally distributed about the value of the population function of each day. The standard deviations of these theoretical normal distributions would tend to increase with the age of the worms, in proportion to the increasing length of the worms. This increasing standard deviation considered for the entire curve is designated as the standard deviation of the function.

Schultz (1930) derived a general equation for the standard error of the function of the logistic curve which is an unbiased estimate of the standard deviation of the function for the population. If sufficient data were available to provide adequate samples of the population for each day, this standard error of the function could be used to establish confidence bands about the theoretical curve. Such bands would be useful for testing the hypothesis that a given worm or group of worms came from the same population represented by the theoretical curve. For example, it would be possible to determine the probability that

a sample of worms from an immune rat came from a population similar to that of worms from non-immune rats. Similar probability tests could be used to determine if the growth of the worms in other species of rodents differed from that which occurs in the normal host, the cotton rat.

Because of the many elements of variability about the curves fitted in this study as well as the inadequate representation of the complete growth cycle, it was felt that the precise fit required for calculating the standard error of the function by the Schultz method was not warranted. It therefore seems impractical to set up confidence bands about the fitted curves. Tests of significance must be based on the standard errors of the observed day means instead of on the theoretical values of the fitted curves.

As mentioned above the standard errors of the day means are presented in table 1. There is a tendency for the standard errors to increase with the age of the worms. It is difficult, however, to make precise estimates of the increase in the standard error associated with the increasing age of the worms because of the unequal number of rats and worms at the various ages.

From the standard errors of the day means the significance of differences between the mean length of worms of the same age from two different types of hosts can be determined. For example, the means of the 24 day old worms harbored in white mice were compared to the means of the 24 day old worms from non-immune cotton rats by the t test for unequal sample variances (Dixon and Massey, 1951). The ten white mice had 72 female worms with a mean of 8.84 $\pm$ 1.02 mm., and 72 male worms with a mean of 7.41 $\pm$ .77 mm. The mean of the 531 female worms in the 27 cotton rats was 9.41 $\pm$ .10 mm. and the mean of the 478 male worms was 7.41 $\pm$ .26 mm. There was no significant difference between the mean lengths in either sex of worms from these species of rodents at 24 days as far as can be determined from the data available.

Discussion and Summary. Any study of growth is based on certain measurements taken over a period of time. The type of measurements that are made depend on the main purpose of the study and the characteristics of the organism to be studied. Since growth of any organism refers to a certain change within a period of time, it is necessary to keep the environmental conditions of

the organism consant throughout the period of measurement. In some cases, the environment can be controlled in terms of diet, temperature, and humidity but variations in the measurements can still be observed because of the inherent differences between individual organisms. In other organisms, such as parasites, it is impossible to control the environmental conditions. The parasite needs its host to exist, therefore the variability observed in studies of parasitic growth is even more complex than that seen in the free-living animal whose environment can be controlled. In other words, any study of growth, whether of a free-living animal or a parasite, must take into consideration various components in the variability of the measurements. If no analysis of the variability is attempted, false conclusions may be drawn from the observed data.

Many equations have been used to represent the course of growth of organisms, whether the measurements are of length or weight, but few people have considered the variability about the fitted curves. Zucker, *et al.* (1942) formulated a log-reciprocal equation to represent the weight-age relationship in post-weaning albino rats. This equation has the characteristic sigmoid shape and thus a point of inflection. Zucker, *et al.* disagree with Brody (1927) on the use of the point of inflection as a means of comparing two growth curves. Brody stated that the point of inflection was a good means of comparing the growth of two organisms, but Zucker, *et al.* found that the point of inflection in rats could be varied by the rat's diet, which means that the point of inflection is not a stable basis for comparison. Zucker, *et al.*, however, compared their different curves only by the relatively inaccurate method of visual estimation of differences.

Since growth curves are usually used for comparative purposes some method of comparing two theoretical curves is necessary. Newcombe, *et al.* (1949) studied the relationship between length, width, weight and thickness of the Blue Crab. They were not measuring the absolute increase of these measurements with age but their relationship with one another. They followed Huxley (1932) in using a constant differential growth ratio $Y = a X^b$ to represent these growth relationships, where Y is one of these dimensions and X is another. They were interested in the difference between the growth of the male and female

crab in terms of these inter-relationships. They compared the growth in the male and female by testing the significance of differences between the two regression of coefficients (b) by Student's t test, after fitting the logarithmic form of Huxley's equation by linear regression. In fitting the logarithmic form by a regression equation, Newcombe, *et al.* were using the method of least squares which gives equal weight to all of their observations. Kettle (1948) analyzed the growth curves of mosquitoes in essentially the same manner as Newcombe, *et al.* A statistical evaluation of growth curves was made by Weil (1947), who felt that the customary practices of visual estimations of differences and the comparison of means by the t test were inadequate for comparing two sets of growth data. He suggested that the data be expressed as frequency distributions. As an example, Weil took weekly weighings of rats over a period of time. All of these weights were plotted as a frequency distribution, one for male rats and one for female rats regardless of the age. Then he compared the weights of male and female rats by a chi square test for differences between the two distributions. This chi square test would determine the significant difference between male and female rats for the specific time interval chosen, but the size of the selected time interval and its location on the complete growth cycle would greatly influence the results of the test for significant differences. None of these methods were applied to the filarial worm data since the methods were felt to be an unsatisfactory means for comparing two growth curves.

The main purpose of this study was to establish a basis for comparing the growth of the worms in rats immunized in various ways with the growth of worms in non-immune rats. Since the experimental method of infecting the rats is a very time consuming and expensive process, the ideal method of comparing the growth of the worms in the two types of hosts would be to compare the observed mean length of a sample of worms from immune rats with the corresponding theoretical value on the curve of worms from non-immune rats. Such comparisons would reduce the number of experiments since parallel experiments in immune and non-immune rats would not have to be run for each day to determine if there was a significant difference in worm length. No method was found, however, which would test the

difference between an observed point and a theoretical point on a curve.

Because comparisons could not be made between observed and theoretical points, the only remaining basis for testing for significant differences was the observed day means. The use of day means for comparative purposes necessitates having two samples of worms of the same age. Numerous such experiments would be necessary to determine the critical period at which the different rate of growth begins in the immune rat. Therefore, this test for differences between day means from two different types of hosts is unsatisfactory in the practical sense.

The results of the analysis of worms of the same age pointed out the existence of sources of variability which had not been fully considered in previous studies. Most parasitologists have neglected these types of variability in their experimental design and thus in analyzing their results. The significant effect of the age and sex of the rats observed in worms of the same age necessitates a change in the design of future experiments. In the past more consideration has been given to the worm variability than to the rat variability. Macdonald and Scott (1953) in their parallel experiments between immune and non-immune rats used larvae from homogeneous groups of mites fed simultaneously on the same rat. The experimental rats used in this particular study of immunity in the cotton rats were of approximately the same age but they were sometimes of different sex. It is quite possible that the sex of the host rat could camouflage the true difference between the length of the worms in immune and non-immune rats. In the experiments of Macdonald and Scott the differences leading to the principal conclusions regarding immunization by live worms were very large. Any effect of the sex of the rat would, therefore, have been relatively small and unimportant. Differences resulting from immunization with dead material were considered small and inconclusive by these authors, but might have been conclusive if all other factors of variability could have been excluded. Further experiments along these latter lines should be planned so as to eliminate completely any possible effect of the sex and age of the rat.

In summary, few specific conclusions can be drawn from a study of this type but the method of the general analysis should

provide a basis for future studies of the growth of parasites. The results of the analysis of variability should serve as a warning to parasitologists working with any phase of parasitic growth. The parasitologist must keep in mind the fact that the variation which he observes in his samples could very well be due to the influence of the host on the parasite and not just due to random variation of the parasite itself.

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THE METABOLISM OF ALCOHOL*

W. W. Westerfeld

Alcoholism has been described as "obviously a metabolic disease," but it must be admitted that we still know very little about how alcohol metabolism could be involved in the problem of alcoholism. The purpose of this communication is not merely to review our present knowledge of alcohol metabolism, but to point out some of the gaps and inconsistencies in our present knowledge. The probable mechanism of alcohol metabolism, on the basis of what is known and what can reasonably be inferred, is shown in figure I. This scheme shows the oxidation of alcohol to acetaldehyde, the conversion of the latter to acetyl CoA, either directly or by way of acetic acid, and finally the complete burning of acetyl CoA through the citric acid cycle.

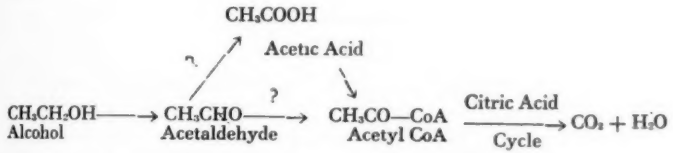

Fig. I. The probable mechanism of alcohol metabolism.

Conversion of Alcohol to Acetaldehyde. The first step, *i.e.*, the conversion of alcohol to acetaldehyde, seems to be on fairly solid grounds. It is the most reasonable possibility from a chemical standpoint, and the only known enzymes capable of metabolizing alcohol convert it to acetaldehyde. In most species the blood acetaldehyde is elevated very substantially after taking alcohol. There are two points, however, which need further exploration. One is the relative importance of each of the two known enzymes which could be involved in this reaction *in vivo:* catalase and alcohol dehydrogenase (Lutwak-Mann; Bonnichsen and Wassen; Theorell and Bonnichsen; Bonnichsen). Keilin and

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Hartree originally showed that catalase, in the presence of peroxide, will oxidize alcohol to acetaldehyde. Since then there have been numerous "educated guesses" about the role of catalase in alcohol metabolism, which have been based upon such factors as the shape of the alcohol disappearance curve, the relative rates of oxidation of methyl and ethyl alcohol, the inhibition of methanol metabolism by ethanol, etc. Based upon such considerations, it seems fairly certain that the catalase mechanism is not of major importance in alcohol metabolism. Jacobsen (1952) has suggested that it may be involved sufficiently to account for the minor deviations from the rectilinear curve of alcohol disappearance occasionally observed. Bartlett has marshalled the arguments against the involvement of catalase in this reaction, and has further demonstrated that the catalase present in crude particulate structures has no peroxidase-like activity. The overall result would be the same whether the alcohol was oxidized to acetaldehyde by means of alcohol dehydrogenase or by means of catalase. However, there is little prospect of correlating alcohol metabolism with the function of alcohol dehydrogenase (Batteli and Stern; Hirsch; Kerner, and Westerfeld) if catalase is also involved in this reaction to any significant extent.

The second point in relation to the conversion of alcohol to acetaldehyde is that the dog does not have an appreciable elevation in blood acetaldehyde after alcohol administration (Westerfeld, *et al.*, 1943; Newman, 1950; Hulpieu, *et al.*, 1954). The dog seems to be unusual in this respect since the rat (Lester and Greenberg), the human (Hald and Jacobsen), and the rabbit (Larsen) all have significant amounts of acetaldehyde in the blood during alcohol metabolism. A species difference of this nature does not necessarily imply that the dog has a fundamentally different mechanism of alcohol oxidation which never yields free acetaldehyde, but we should at least be aware of the fact that there is something different about alcohol metabolism in the dog as compared with the human and other species. Much of our information about the rate as well as other aspects of alcohol metabolism has been obtained with the dog. At the present time, no species difference in other aspects of alcohol metabolism can be attributed to this difference in blood acetaldehyde response.

The reason for an elevated blood acetaldehyde after alcohol administration in any species is not clear. At first glance it would seem obvious that if alcohol were converted to acetaldehyde, the latter should appear in the body fluids; but this is not necessarily true. The equilibrium between alcohol and acetaldehyde in an *in vitro* system is greatly in favor of the alcohol (Negelein and Wulff) by a ratio in the order of 1300:1. In those species which accumulate acetaldehyde after receiving alcohol, the blood alcohol: acetaldehyde ratio is of the order of 130:1 (Raby). In other words, there is 10 times as much blood acetaldehyde present as would be expected from the *in vitro* equilibrium figures. This point alone may not be too significant since the ratio could be influenced by the formation of complexes between the enzyme and reduced DPN (Kaplan and Ciotti; Theorell and Chance; Hayes and Velick; Theorell and Bonnichsen). However, the rate of acetaldehyde metabolism is much faster than the rate of alcohol metabolism in all species studied, and all of the acetaldehyde formed by the oxidation of alcohol should theoretically be destroyed as rapidly as it is formed. How and why it accumulates to the extent that it does is not at all clear.

Acetaldehyde in Alcoholism. So far as the human is concerned, acetaldehyde is clearly a substance which has to be taken into consideration as a part of the alcohol picture (Mendeloff). The problems of alcohol really involve both alcohol and acetaldehyde as "foreign agents." Acetaldehyde is not a normal metabolite of the usual foodstuffs (Jacobsen, 1950) and is not found in the body in significant amounts except after the administration of alcohol. Antabuse pratially blocks the metabolism of acetaldehyde (Jacobsen, 1952) and produces its typical reaction only after the administration of alcohol.

Which effects of alcohol are due to alcohol, *per se*, and which are due to the acetaldehyde or a combination of alcohol and acetaldehyde (MacLeod; Hald, *et al.*, 1952)? Physiologically they are quite different substances. Alcohol can be tolerated in relatively large amounts (Parker, *et al*), with blood levels of several hundred mg. percent being perfectly compatible with life. Blood levels of 50 mg percent acetaldehyde or less will produce death. Alcohol is metabolized rather slowly along a straight line curve with time, whereas acetaldehyde is metabolized very

rapidly and exponentially (Lubin and Westerfeld; Hald and Larsen; Westerfeld, *et al.*, 1949.)

Acetaldehyde will produce symptoms in the rat which could be interpreted as intoxication (Stotz, *et al.*), but the symptoms of intoxication cannot be related to blood acetaldehyde rather than blood alcohol for two reasons: 1) the dog can be quite intoxicated from alcohol without having any significant amount of acetaldehyde in the blood, and 2) other species do not have sufficiently high blood acetaldehyde levels after alcohol administration for the acetaldehyde to be responsible for the intoxication symptoms (MacLeod).

Dr. Stotz measured the acetaldehyde: alcohol ratios in different people after alcohol administration, and found that chronic alcoholics tended to have a lower ratio and abstainers tended to have a higher ratio than did the moderate drinkers. This would suggest that part of the conditioning process toward alcohol (Newman and Card; Newman and Lehman; Newman, 1941; Levy) might be the development of a better mechanism for handling acetaldehyde. These preliminary studies by Stotz were never published because they were not done on a large enough series. They are mentioned here to emphasize that studies on acetaldehyde, *per se*, are an inherent part of the alcohol picture, and that this is an area which has been developed very little.

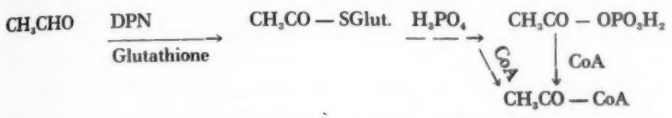
Acetaldehyde Metabolism. The pathway of acetaldehyde metabolism is not yet established. There is no doubt that some of it, at least is eventually converted to acetyl CoA. Isotopically labeled alcohol can be found in the acetyl group of an acetylated amine (Bernhard; Bloch and Rittenberg). The acetyling agent has been pretty well established as acetyl CoA (Chou and Lipmann; Lynen, *et al.* 1952), so the overall conversion of alcohol to acetyl CoA can be accepted as factual. The only major point in doubt is whether the acetaldehyde is converted to acetyl CoA by a direct mechanism, or whether acetic acid is an intermediate in the process.

There are at least six enzymes found in the body which are capable of utilizing acetaldehyde as a substrate:

1) Aldolase: (Meyerhof, *et al.*)

$$CH_3CHO + H_2PO_3 - O - CH_2 - CO - CH_2OH \longrightarrow$$
$$H_2PO_3 - O - CH_2 - CO - CHOH - CHOH - CH_3$$

2) Glyceraldehyde—3—phosphate dehydrogenase: (Harting; Racker and Krimsky; Harting and Velick; Lipman; Stadtman).



3) Carboxylase: (Green, *et al.* Berry and Stotz; Jagannathan and Schweet; Smith and Hendlin).

$$CH_3CHO + CH_3COCOOH \xrightarrow[-CO_2]{\text{DP–Thiamin, Lipoic Acid}} CH_3 — CHOH — CO — CH_3$$

4) Xanthine Oxidase: (Ball; Booth; Corran, *et al.*; Mackler, *et al.*; Richert and Westerfeld).

$$CH_3CHO \xrightarrow[O_2]{Mo,\ B_2,\ Fe,} CH_3COOH$$

5) Aldehyde oxidase: (Carpenter; Knox; Mahler, *et al.*).

$$CH_3CHO \xrightarrow[O_2]{Mo,\ B_2} CH_3COOH$$

6) Aldehyde dehydrogenase (Mutase) (Racker; Dixon and Lutwak-Mann; Battelli and Stern; Parnas; VonEuler and Brunins)

$$CH_3CHO \xrightarrow{DPN} CH_3COOH$$

$$CH_3CHO + CH_3CHO \xrightarrow{DPN} CH_3COOH + CH_3CH_2OH \quad + \text{Alc. dehydrogenase}$$

$$CH_3CHO + CH_3COCOOH \xrightarrow{DPN} CH_3COOH + CH_3CHOHCOOH \quad + \text{Lact. dehydrogenase}$$

The problem is to determine which, if any, of these enzymes are involved *in vivo*. The aldolase, carboxylase, and glyceraldehyde-

3-phosphate dehydrogenase are abundant in muscle, whereas the major part of acetaldehyde metabolism takes place in the liver (Lubin and Westerfeld; Hald, *et al.*, 1949). This alone is inadequate to eliminate them completely from further consideration, since they might be involved as liver enzymes. However, it tends to make their participation less probable since there is no obvious reason why the same enzyme would be active in liver and inactive in muscle. Carboxylase can definitely be ruled out on other grounds (Lubin and Westerfeld; Greenberg; Berg, *et al.*).

It is likely that whatever enzyme(s) is primarily responsible for acetaldehyde metabolism *in vivo* is not only very active in liver, but is inhibited by antabuse (Jacobsen, 1952), and is dependent upon some unidentified dietary factor (Westerfeld, *et al.*, 1949). Xanthine oxidase, aldehyde oxidase and aldehyde dehydrogenase are abundant in liver, and are all inhibited by antabuse. However, xanthine oxidase is not particularly sensitive to this inhibitor (Richert, *et al.*), and is not responsible for acetaldehyde metabolism, since the latter is not influenced appreciably when this enzyme is removed from the liver by a low protein diet (Westerfeld and Richert). The aldehyde oxidase (Kjeldgaard; Schwartz and Kjeldgaard) and aldehyde dehydrogenase (Graham) are both inhibited by very low concentrations of antabuse. Like xanthine oxidase, the aldehyde oxidase is a molybdenum-enzyme (Mahler, *et al.*), and over ⅔ of the Mo can be removed from the liver without affecting acetaldehyde metabolism (Westerfeld and Richert); the role of aldehyde oxidase in acetaldehyde metabolism has not been studied directly. No information about the role of aldehyde dehydrogenase is available, but the classical mutase action involving two molecules of acetaldehyde does not take place *in vivo* because alcohol is not formed after acetaldehyde administration (Westerfeld *et al.*, 1943; Jacobsen, 1952). However, the dehydrogenase could still be involved in a direct reaction or in a coupled oxidation-reduction reaction with other substrates.

Both of the known liver enzymes which could be involved in acetaldehyde metabolism *in vivo* convert it to acetic acid rather than acetyl CoA. In liver slices metabolizing alcohol, acetic acid accumulates (Leloir and Munoz), and rat liver mitochondria

oxidize acetaldehyde to acetic acid without the intervention of CoA (Walhenstein and Weinhouse). Acetic acid does not accumulate during alcohol metabolism *in vivo* (Himwich, *et al.*; Friedemann and Kimeciak) either because it is not formed directly or because it is further metabolized as rapidly as it is formed.

Microorganisms can form acetyl CoA from acetaldehyde without the intermediation of acetic acid (Burton and Stadtman; Pinchot and Racker). A purified yeast enzyme will oxidize acetaldehyde to acetic acid without involving CoA, (Black; Seegmiller), but yeasts can oxidize acetaldehyde under conditions where acetic acid is not oxidized (Lynen; Black and Hutchens) presumably by the direct formation of acetyl CoA from acetaldehyde (Lynen and Reichert). The possibility of a direct conversion of acetaldehyde to acetyl CoA in liver by means of the glyceraldehyde phopshate dehydrogenase or some as yet unknown enzyme is enhanced by the finding of Brady and Gurin that acetaldehyde is a better source of fatty acids than is acetate. If acetic acid is formed from acetaldehyde *in vivo*, there is no difficulty about its subsequent conversion to acetyl CoA by known metabolic reactions.

Acetyl CoA Metabolism vs. Alcohol. Once the acetyl CoA is formed, we can readily conceive of it being metabolized via the citric acid cycle (Bloch; Stern, *et al.*) to CO_2 and H_2O. Labeled alcohol does contribute to the two-carbon pool and can give rise to labeled fatty acids or cholesterol (Curran and Rittenberg; Bloch and Rittenberg), but no large portion of the ingested alcohol is diverted to such pathways; most of the alcohol is burned to CO_2 (Bartlett and Barnet; Dontcheff).

If alcohol is metabolized predominantly via acetyl CoA, then the latter is the common meeting point for the breakdown of alcohol and fatty acids (Fig. II). Carbohydrate apparently supplies the oxaloacetate for combination with the acetyl CoA to form citrate, and it seems probable that the need for carbohydrate metabolism to prevent ketosis is related to this reaction. This would also explain the increased carbohydrate metabolism which accompanies alcohol metabolism (Westerfeld, *et al.*, 1942). On this basis, alcohol should be just as ketogenic in the diabetic as are the fatty acids since large amounts of acetyl CoA

would be diverted to acetoacetic acid in this situation. Increased amounts of ketone bodies would not result from alcohol if the latter simply replaced fatty acids as the source of acetyl CoA, but in this sense, at least, alcohol metabolism is more akin to fat than carbohydrate metabolism.

If this is the correct mechanism for handling alcohol, then both alcohol and fats might be expected to be quivalent in their physiological relationships because both would be metabolized to the

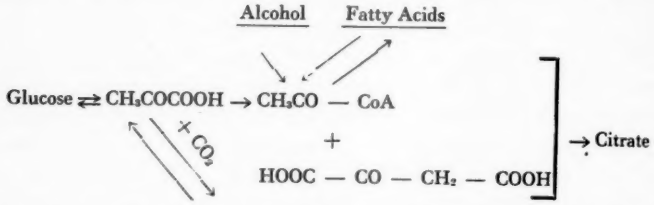


Fig II. Alcohol in Relation to Fat and Carbohydrate Metabolism.

same intermediate. However, the evidence indicates that fat and alcohol are not equivalent nutritionally. Large amounts of fat will allow an animal to grow better than will large amounts of alcohol (Westerfeld and Doisy; MacDowell; Mallov; Elhardt). Isocaloric alcohol is therefore not equivalent to fat in so far as growth and body weight are concerned. This may not be difficult to explain. If the organism converts alcohol to fat before depositing it, energy will be required to effect these transformations. Fat fed as such would not require the same expenditure of energy in order to be deposited because it need not be broken down first.

If acetic acid is an intermediate in alcohol metabolism, energy (as ATP) will be required to convert it to acetyl CoA. In the breakdown of fatty acids, ATP is not required for the formation of each acetyl CoA unit; once the reaction is started, acetyl CoA units can be formed from all of the carbons of the fatty acid without a further expenditure of ATP energy. This is not of major importance, however, since the amount of energy spent in the formation of acetyl CoA is relatively small in comparison with the large amount of energy derived from the further metabolism of the acetyl CoA.

Overall Aspects of Alcohol Metabolism. The most striking fact about the overall metabolism of alcohol is that blood alcohol

disappears along a straight line relationship, while most substances disappear metabolically as an exponential function with time. There has been some questioning of the rectilinear nature of alcohol metabolism, but excepting the exponential phase at very low blood levels (Marshall and Fritz), its disappearance is certainly much closer to a straight line than to an exponential relationship (Marshall and Fritz; Jacobsen, 1952). Since the metabolism of alcohol proceeds at approximately a constant rate regardless of the blood concentration (Loomis; Newman and Cutting, 1935; Newman, *et al.*, 1937; LeBreton, 1935; Eggleton, 1940; Southgate), the enzyme (or enzymes) involved in the rate-determining reaction must be saturated by relatively low concentrations of alcohol, and higher concentrations of substrate are without additional effect. This agrees with the kinetic data for purified alcohol dehydrogenase (Theorell and Bonnichsen), although the concentration of alcohol required to saturate the enzyme *in vivo* may be 10-fold higher than *in vitro* (Vitale, *et al.*, 1953). The rate-limiting reaction in alcohol metabolism, at least in the dog, is the conversion of alcohol to acetaldehyde, since: a) the rate of acetaldehyde disappearance is much more rapid than the rate of alcohol disappearance (Westerfeld, *et al.*, 1943); b) acetaldehyde administered during alcohol metabolism can be metabolized completely without affecting the rate of alcohol disappearance; and c) when acetaldehyde metabolism is partially blocked by antabuse (Newman and Petzold), there is not a corresponding decrease in the rate of alcohol metabolism (Loomis).

In the dog the maximum rate of alcohol metabolism appears to be in the range of 20–25 mg. percent per hour decrease in the blood, and the minimum rate observed is 8 to 10 mg. percent per hour. Many dogs are normally in the range of 10–15 mg. percent per hour, and the latter can be increased up to the maximum rate by such things as insulin plus glucose. This was first demonstrated by Widmark (1935) for the insulin effect (Newman and Cutting, 1948), and is essentially the same type of response obtained with fructose (Pletscher, *et al.*), alanine (Widmark, 1933, LeBreton, 1934) and pyruvate (Westerfeld, *et al.*, 1942). Both the insulin effect (Loomis; Clark, *et al.*) and the pyruvate effect (Hulpieu, *et al.*) 1948; Gregory, *et al.*; Greenberg; Kinard, *et*

al.; Jacobsen, 1952) have been controversial. Many of the inconsistent results can be attributed to two factors: 1) results obtained with dogs cannot be assumed to apply to rats, and 2) these agents are effective in the dog only when the initial rate of alcohol metabolism is less than maximal; the magnitude of their effect depends upon the existing rate of alcohol metabolism at the time they are administered. The lack of response by rats to pyruvate was reported initially (Westerfeld, *et al.*, 1942) and has since been observed by others (Vitale, *et al.*, 1954; Bartlett and Barnet). The pyruvate effect was obtained with dogs that had minimal rates of alcohol metabolism at the time the pyruvate was administered, and these low rates were thereby increased to the maximum rates. Since then, we have tested pyruvate in two dogs whose initial rates of alcohol metabolism were 22–25 mg. percent per hour, and found no effect. Most of the negative results reported for pyruvate have been obtained with dogs having initial rates of alcohol metabolism in the range of 20 mg. percent per hour (Gregory. *et al.*; Hulpieu, *et al.*, 1948; Kinard, *et al.*)

A possible explanation for these results is the following. The maximum rate of alcohol metabolism is limited by the amount of alcohol dehydrogenase present, but the maximum rate is not achieved by the action of the enzyme alone. Some additional factor is coupled with the enzymatic oxidation of alcohol to acetaldehyde in order to speed up the reaction, and in the relative absence of this factor, minimal rates of alcohol metabolism are observed. It has been suggested (Westerfeld, *et al.*, 1943) that the factor involved in this reaction is a coupled oxidation-reduction reaction between the alcohol and some other substrate such as pyruvate. From the oxidation reduction potentials of the systems involved, it is obvious that the oxidation of alcohol to acetaldehyde by a DPN-enzyme cannot proceed very far unless the acetaldehyde is removed and the DPN is regenerated; the rates at which these latter reactions are effected can be of greater importance to the overall rate of alcohol metabolism than the amount of enzyme present. On such a basis, the rate of alcohol metabolism existing at any given time would be determined by the amount of coupling substrates (such as pyruvate) present. Insulin plus glucose, fructose or alanine would increase the

amount of pyruvate available for such a reaction, and the magnitude of the pyruvate effect would be dependent upon the extent to which this reaction was already taking place prior to its administration. The acidosis following alcohol ingestion is not due to acetic acid (Eggleton, 1946) but is related to the increased blood lactate which presumably arises from this coupled reduction of pyruvate. The alcohol addict appears to have an adapted carbohydrate metabolism with respect to the formation of pyruvate from glucose (Varela, *et al.*), since higher blood pyruvate levels are found in addicts after glucose administration. It could also be concluded that the rat not only oxidizes alcohol more rapidly than the dog, but that the coupled reaction is functioning maximally in the rat under normal circumstances. Fasting (Vitale, *et al.*, 1953; 1954) or feeding a low protein diet (Kerner and Westerfeld) would decrease the rate by removing alcohol dehydrogenase from the liver.

This coupled reaction could explain another puzzling phenomenon in alcohol metabolism. Although the blood alcohol falls rectilinearly, continuous infusion studies have indicated that alcohol is metabolized faster when the blood alcohol level is high. These results suggest that the initial rate of alcohol metabolism is "triggered-off" by the blood alcohol concentration, but once started it proceeds at a constant rate thereafter (Newman, *et al.*, 1937; Newman, 1947). There is no obvious way in which the simple oxidation of alcohol by alcohol dehydrogenase could be "triggered-off" by the alcohol concentration, but the simultaneous rate of carbohydrate metabolism might be influenced by a hormonal stimulus (epinephrine? (Christensen)) which could vary with the amount of alcohol or acetaldehyde in the body fluids. The effect of blood alcohol levels on the rate of alcohol metabolism would then be indirect, mediated through its influence on carbohydrate metabolism, and dependent upon the coupled oxidation-reduction reaction between alcohol and carbohydrate intermediates.

Alcohol Metabolism in Brain. There is general agreement that the liver is the organ primarily responsible for alcohol metabolism. In some species the kidney may also play a minor role (Mosoro, *et al.*; Bartlett and Barnet). Whether the brain can oxidize alcohol at all is questionable. Earlier studies (Himwich, *et*

al.; Wortis) indicated that the respiratory quotient of excised brain falls slightly in the presence of alcohol, and that the O_2 consumption may be stimulated initially (Robertson and Stewart); O_2 consumption is inhibited by higher concentrations (Buchel; Fuhrman and Field). An oxidation of alcohol by the brain is not adequate to sustain the brain, affect the A-V oxygen or glucose difference (Loman and Myerson) or restore consciousness during hypoglycemia (Goldforb and Wortis), but this is also true of known substrates for brain metabolism. More recent studies with isotopic alcohol (Bartlett and Barnet; Mosoro, *et al.*) have shown no metabolic activity by excised brain tissue toward this substrate; there was no production of isotopic CO_2 or substances (fatty acids and cholesterol) commonly derived from the 2-carbon acetyl unit in other tissues. This is difficult to reconcile with the demonstration of small amounts of an "alcohol dehydrogenase" in brain by Dewan, since this enzyme converts alcohol to acetaldehyde, and the latter can be converted to citrate by brain tissue (Berry and Stotz). Berry and Stotz also showed that acetaldehyde gives increased amounts of citrate, acetoin, and acetyl choline in excised brain, while alcohol increases only the acetyl choline fraction. From the method used by Dewan for the preparation of brain alcohol dehydrogenase, this enzyme appears to be associated with the insoluble fraction, and in this respect at least is quite different from the alcohol dehydrogenase of liver and yeast. We can assume that a minor mechanism is potentially available in brain for the oxidation of alcohol to acetaldehyde, but the utilization of this reaction *in vivo* seems improbable, and even if small amounts of alcohol were oxidized to acetaldehyde in brain, it would be of little importance.

The role of alcohol in brain metabolism cannot be dismissed simply because the brain itself has a very limited or negligible capacity for oxidizing alcohol to acetaldehyde. It can metabolize acetaldehyde (Hine, *et al.*), and the latter is supplied to the brain during alcohol metabolism via the blood stream. There is some evidence that different tissues in the body metabolize acetaldehyde by different mechanisms (Westerfeld), and the possibility that acetaldehyde is metabolized in brain by a mechanism which is qualitatively different from liver needs further exploration. It is evident that brain is a unique tissue in relation to the

alcohol problem, but it is not yet known whether the pharmacological effects are related to alcohol or to its metabolites.

Fatty Livers. Nutritional studies during the past 25 years have pretty well dispelled the idea that alcohol is an hepatic poison. There is no reason to doubt that the nutritional deficiencies seen in chronic alcoholics are largely due to a very poor diet in which the main source of calories consists of alcohol. The cirrhosis of alcoholism can be attributed to the low-protein, low-choline, low-vitamin diet consumed (Lowry, *et al.*; Chaikoff, *et al.*) but it is also possible that alcohol acts like dietary fat in increasing the choline requirement. This point has been considered by a number of laboratories, with conflicting results (Best, *et al.*; Klatskin, *et al.*, 1951; 1954; Forbes and Duncan, 1950, Mallov).

The manner in which choline prevents fatty infiltration of the liver is by no means established. It was previously assumed that the choline was needed for the formation of phospholipids, which in turn were needed for the transport of fat out of the liver. However, the phospholipid components of the fatty, choline-deficient rat liver and all of its particulate fractions are perfectly normal (Spiro and McKibbin). A choline deficiency may simply depress the rate of phospholipid turnover, but we are also beginning to think of choline as a possible factor in the metabolism of the 2-carbon unit, acetyl CoA (Artom). If this be true, then the alcoholic would not only be getting a choline-poor diet, but would have need for relatively large amounts of choline in the metabolism of this 2-carbon substance. Cirrhosis would still remain a nutritional deficiency, but it would be a deficiency partly induced by the increased requirement for choline in alcohol metabolism.

Osmotic Pressure. There is a potential relationship between alcohol and the osmotic pressure of body fluids which has not been explored adequately. Only substances present in reasonably high concentrations and possessing a relatively small molecular weight could exert any major effect on the osmotic pressure of the body fluids. A concentration of 200 mg. percent alcohol would exert an osmotic pressure of approximately 1 atmosphere; this is about 15 percent of the total osmotic pressure of plasma. If it is true that the organism attempts to maintain a constant osmotic

pressure in its body fluids, then alcohol should have enough of an osmotic effect so that some counter-balancing action should be brought into play. The alcohol cannot be excreted selectively, nor can it be metabolized rapidly. Hence the osmotic pressure of the body fluids must either remain above normal throughout the period that alcohol is being metabolized or some other component of these fluids (such as NaCl) must be selectively removed when alcohol is introduced. If some component is selectively removed while the blood alcohol concentration is high, then after the alcohol has been metabolized, there will be a deficiency of this component and a decreased osmotic pressure of the body fluids. Alcohol should, therefore, elicit different osmotic responses when its concentration in the blood is rising and falling, and it is entirely possible that the "pick-me-up" drink following an alcoholic bout is the quickest way of restoring the osmotic pressure of the body fluids.

The literature on the diuretic action of alcohol and the effect of alcohol on the excretion of NaCl is both voluminous and confusing. Some of this confusion is related to the different response by the animal to rising and falling blood alcohol levels (Haggard, *et al.*) and some to differences in the concentration of alcohol administered and the timing of the experiments. The washing out of urea from the body fluids as a result of alcohol administration (Melka) could be related to this osmotic effect since some species of fish maintain the osmotic pressure of their body fluids with high concentrations of urea, and mammals have high blood concentrations of urea during the salt deficit of adrenal insufficiency. Alcoholics tend to be hypoadrenal (Mann) and to have a low plasma NaCl concentration; the latter has been reported (Silkworth and Texon) to be related to the craving for alcohol. The loss of adrenal ascorbic acid and cholesterol after alcohol (Forbes and Duncan, 1951, 1953; Gley) is indicative of adrenal stimulation, and the adrenal is part of the posterior pituitary-adrenal axis which would be expected to be involved in osmotic pressure adjustments.

There is sufficient information currently available to suggest that the osmotic and electrolyte effects of alcohol may be of tremendous importance to an understanding of the alcohol problem. The imbalance in Na and Cl excretion (Kennard, *et al.*),

the suggested use of ACTH in the treatment (Smith) and the recent studies relating the tremors of DT's to an inadequate blood level of magnesium further emphasize the need for a more adequate development of this relationship of alcohol to fluid and electrolyte balance.

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(Titles are omitted in interests of economy.)

EFFECT OF THIAMINE ON THE GROWTH, SURVIVAL, SENESCENCE AND MORPHOLOGY OF *TETRAHYMENA PYRIFORMIS (Y)*[*]

W. J. Wingo,[1] L. S. Lockingen,[2] and L. E. Cameron

Recently the authors had occasion to autoclave five gallon lots of one per cent proteose-peptone-mineral salts medium (Wingo and Cameron, 1952) for use in growth experiments with *Tetrahymena pyriformis (Y)*,[3] in a constant flow system (Browning and Lockingen, 1953). In order to insure sterility, autoclaving was carried out at 15 pounds pressure for three hours. A finely divided amorphous precipitate formed in the five gallon container; and, since the constant flow system used depended upon a siphon and constant speed pump for delivery of fresh medium to the growth flask, this precipitate was not available to the organisms. Very scanty growth resulted whereas smaller vessels of the same medium, autoclaved for only 15 minutes, supported luxuriant growth in the presence of small quantities of the precipitate.

Since the medium was prepared and handled in the same way as in earlier experiments except for the protracted autoclaving, it was thought that some thermolabile component was being destroyed during sterilization. Thiamine was considered first, since Farrer (1941), Booth (1943), and Beadle, Greenwood, and Kraybill (1943) have reported extensive destruction of thiamine by boiling at pH's near that used (7.3). However, the inclusion of high concentrations of thiamine in other large lots of media did not significantly increase growth in the flow system. The fortified medium in small flasks produced extraordinarily dense cultures; after a phase of rapid senescence, these cultures exhibited

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[3] This is the same organism called *T. geleii Y* in earlier papers from this laboratory. The change in name brings our terminology into accord with that of J. O. Corliss (*Parasitology*, 43: 49–87, 1953).

pronounced "secondary growth" (Wingo and Lockingen, 1954). Experiments were then undertaken to measure these effects under controlled conditions. The results indicate that the thiamine supply in a culture is important, not only in regard to the maximum culture density obtained, but also in regard to the behavior of the cells after the maximum population has been reached.

Experimental

The medium was made as usual (Wingo and Cameron, 1952), and autoclaved at 15 pounds per square inch for 15 minutes. It was then centrifuged free of precipitate and placed in growth flasks of the type described by Browning, Brittain, and Bergendahl (1952) or in large Florence flasks with provision for aeration and sampling without bacterial contamination. The medium was then autoclaved again. In each experiment, a pair of identical flasks were prepared and reautoclaved at the same time. Each of a pair of such flasks was inoculated from the same stock culture; and to one flask of the pair was added, asceptically, a known amount of a separately-prepared and sterilized one per cent solution of thiamine hydrochloride. The unfortified cultures were designated as controls. The cultures were incubated, under dim illumination and constant aeration, in a water bath at 37° C. Samples were taken and counts made by the procedure previously described (Wingo and Lockingen, 1954). Photomicrographs were taken of each sample (at constant magnification) for a record of the morphological changes which occurred.

Results

The results, as regards culture densities in a typical experiment, are presented in figure I.

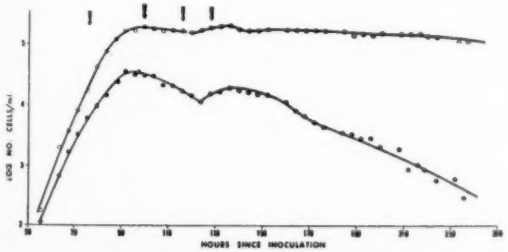

FIG. I. Changes in population with time in cultures of *T. pyriformis* (*Y*) grown without added thiamine (lower curve) and with the addition of 4 mg. thiamine hydrochloride in 250 ml. medium (upper curve). Arrows indicate sampling for photographs at 75.5 hr., 99.5 hr., 115.5 hr., and 127.5 hr. after inoculation.

The larger numbers of cells produced in the culture fortified with thiamine is evident. The death phases of these cultures may be separated conveniently into three stages. In the first stage, the control culture declined rapidly in density whereas the thiamine-fortified culture diminished only slowly in density. In the second stage a marked secondary growth phase was manifested in the control culture as compared to a questionable increase in the numbers in the fortified culture. In the third stage, the control culture "died" at a rapid rate, whereas the fortified culture decreased only very slowly in population.

Marked morphological differences in the cells in the paired cultures were also observed. Once the initial phase of growth had ended, the cells grown without added thiamine became smaller and more nearly spherical until the onset of the phase of secondary growth. At this time, cells of the same shapes and sizes as those seen in the first growth phase again appeared. When the peak of the secondary growth phase had been passed, the cells again became small and round. The cells in the culture fortified with thiamine remained "normal" in shape throughout the period of observation and became larger after the maximum population had been reached. The differences between the cells of the two cultures whose growth behavior is described by the curves in figure I are shown by the photomicrographs in figure II. Unfortunately, the photographs taken after 128 hours were underexposed; however, figure III, taken from a similar pair of cultures at a time considerably past the secondary growth peak of the unfortified culture, shows the general appearance of the cells at this time.

During periods of rapid decline of population in the unfortified culture rapid lysis occurred; the counts included all recognizable cells or major fragments of cells in the fields examined and at these times a significant proportion of the cells counted showed signs of incipient or actual lysis.

Discussion. Johnson and Baker (1943) reported that thiamine promoted the growth of *T. geleii;*[4] they observed secondary growth *in the cultures containing added thiamine.* Their conditions were, however, quite different from those used here; and their secondary growth phase was quite delayed, its maximum

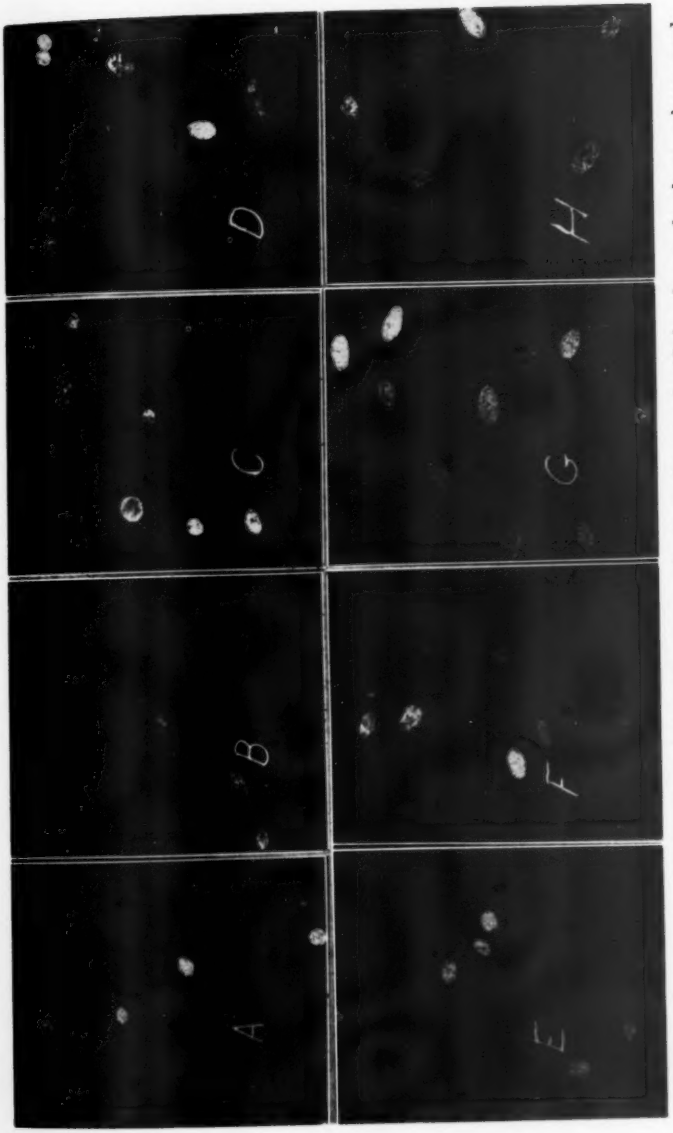

FIG. II. Dark-field photomicrographs (163 X) taken of samples from thiamine-fortified and control cultures whose population-time curves are shown in figure I. (A, B, C, D, control culture at 75.5, 99.5, 115.5, 127.5 hrs.); (E, F, G, H, thiamine-fortified culture at 75.5, 99.5, 115.5, 127.5 hrs.). Note division figure in control at 127.5 hrs.

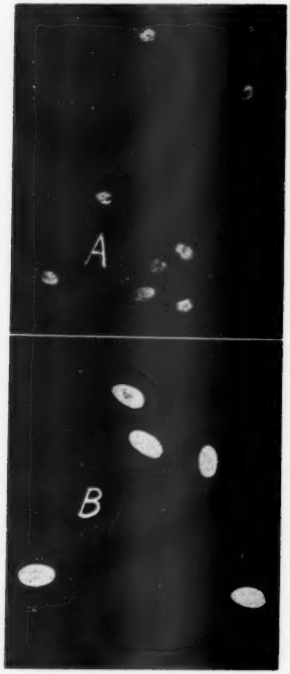

FIG. III. Dark-field photomicrographs taken of another pair (A, control; B, thiamine-fortified) of cultures of *T. pyriformis* (*Y*) at point corresponding approximately to 210 hrs. in figure I.

being reached only after several weeks. Kidder and Dewey (1944) reported the synthesis of thiamine by *T. geleii*[4] *W*,. *and* observed great differences between the maximum population density of cultures grown with and without thiamine or factors facilitating its biosynthesis. They also observed in some circumstances that cultures without thiamine declined more rapidly in numbers than did fortified cultures. Apparently secondary growth did not occur under the conditions they employed.

It would appear that, under the conditions obtaining in the present investigation and in the experiments on secondary growth described earlier (Wingo and Lockingen, 1954), a rela-

[4] *(T. pyriformis.)*

been diminished by a phase of rapid death. (The very slight increase in numbers in the thiamine-fortified cultures which occurred at the time that the marked secondary growth phase occurred in the control culture may represent secondary growth; but if this were the case, it was very much smaller than that in the control cultures.)

The great differences between the sort of secondary growth observed in these and in our earlier experiments (Wingo and Lockingen, 1954) and the kind of secondary growth ordinarily obtained (which consists of a second growth curve superimposed upon the plateau of a first growth curve) make it improbable that the phenomenon described here represents either adaptation in the sense used by Lodge and Hinshelwood (1944) or a mutation of the cells to synthesize a limiting nutrient. Instead it appears probable that in the present case a depletion of thiamine brings about a first death phase and that the secondary growth phase following this rapid decline in numbers occurs through the reutilization of small amounts of thiamine liberated by the lysed cells.

This follows from the known great importance of thiamine in metabolic energy generation and from the necessity of the generation of energy by the cells to perform osmotic work, to maintain ciliary action, etc. If, in the performance of its metabolic functions, thiamine is degraded, the cells grown in a medium relatively deficient in thiamine and relatively abundant in "structural" materials could grow normally only so long as the supply of thiamine was fairly abundant. When substantially all the thiamine had been removed from the medium, growth would entirely cease and the cells could live only by virtue of using the thiamine which they contained. As this intracellular thiamine supply became depleted through "wearing out" in use the cells would no longer be able to carry out the processes of energy generation necessary for their continued survival, and would be expected to die at an approximately exponential rate.

Tittler, *et al.* (1952) have reported that the failure of growth which they observed was probably not due to the pH changes

occasioned by pyruvic acid accumulation, since growth failure also occurred in strongly buffered media. It is equally improbable that pyruvate accumulation *per se* was responsible for the first death phase observed in the "control" cultures in the present study, since the medium was buffered to pH 7.3 with phosphate (0.05M), and since in the many earlier cultures in which pH was measured it was never observed to drop below pH 7.1.

If the small amounts of thiamine contained in the dying cells were (by some process of autolysis) once again made available to the cells which survived one would expect these survivors once again to begin growing until this re-released supply of thiamine had in its turn been depleted. Under these conditions the secondary population peak should in turn be followed by a further decrease in numbers of cells per unit volume. If the relative concentrations of "structural" and "energy releasing" nutrients in the medium were properly adjusted a series of descending growth peaks should be observed. This was not the case in any of the authors' experiments, but the small numbers involved in the later phases of "unfortified" cultures make the counting procedure relatively inaccurate so that such growth peaks might occur but escape detection by the methods used.

If the limiting nutrient (not thiamine) is not "worn out" in fulfilling its metabolic functions and if the thiamine concentration in the medium is relatively large, a higher population will be reached and will be maintained until either the substances oxidized to produce energy or the large thiamine supply are depleted. This course of events appears to have been fulfilled in the "thiamine-fortified" cultures in the present investigation.

It is admitted that the results of this study are of greater use in the framing of hypotheses than in their testing; nevertheless, they strongly suggest that a culture of free-living cells may use up its supply of a growth factor with the resulting death of a large fraction of the total number of cells present and that the tive deficiency of thiamine was responsible for the first phase of rapid death in cultures of *T. pyriformis* (*Y*). Significant secondary growth was never observed unless the numbers of cells had

release of growth factor by the dying cells may lead to appreciable recovery in numbers of normal cells present. It is to be hoped that experiments using synthetic media may lead to an explanation of the phenomena herein reported.

Summary. *T. pyriformis* (*Y*) grown in phosphate buffered one per cent proteose-peptone media which has been autoclaved, clarified by centrifuging and reautoclaved exhibit an early rapid initial death phase followed by a secondary growth at the end of which the number of cells produced is considerably smaller than the original maximum population.

Parallel cultures which differ only in that the medium has been fortified with large quantities of thiamine attain higher maximum populations and remain at high levels of population long after the unfortified cultures are in advanced stages of senescence.

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NINTH ANNUAL SYMPOSIUM ON FUNDAMENTAL CANCER RESEARCH

The University of Texas

M. D. Anderson Hospital and Tumor Clinic

Houston, Texas

March 10, 11, 12, 1955

THE HISTOCHEMISTRY OF CANCER

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M. D. Anderson Hospital and Tumor Clinic

The Bertner Foundation Lecturer:

Dr. George N. Papanicolaou

Cornell Medical School, New York City

NINTH ANNUAL CANCER SYMPOSIUM, 1955

R. Lee Clark, Jr.

James Ewing often remarked that cancer was not one disease but a thousand different diseases. Because of the complexity of the problem, it seems likely that the crucial answers must eventually be found in basic research in biology, biochemistry, pathology, and almost every other phase of medical and physical science. Belief in the necessity and efficacy of basic research resulted in the institution of the Annual Symposium on Fundamental Cancer Research by The University of Texas M. D. Anderson Hospital and Tumor Institute in 1946. Since 1946, nine symposia have been held, each with the purpose of reporting, collating, and evaluating current research projects. At these meetings, the most outstanding investigators have been invited as speakers, thereby affording opportunity for knowledge to be exchanged, discussed, and integrated in an effort to co-ordinate every possible approach to research.

The subjects for earlier symposia have included isotopes in cancer research, protein metabolism in relation to cancer, and aspects of tissue culture in cancer research. This year, over 200 specialists in cancer research registered for a program of three major topics. These included epidemiology, histochemistry of cancer, and a survey of recent developments in cancer research. At the opening session, Dr. John Fertig emphasized the necessity for care in the design of cancer experiments, and described several ways to eliminate error caused by prejudice on the part of the observers and psychological effects on the subjects.

The outstanding single feature of each annual symposium is the presentation of the Bertner Foundation Award, and the Bertner Lecture which is delivered by the Award winner. The Bertner Award was established in 1950 in honor of the late Dr. E. W. Bertner, first acting director of the M. D. Anderson Hospital and first president of the Texas Medical Center. The award is presented annually for outstanding achievement in cancer research. The recipient of this year's award was Dr. George N. Papanicolaou of Cornell University for his research contribu-

tions in exfoliative cytology. Dr. Papanicolaou chose as his subject for the Bertner Lecture, "Reflections on the Evolutionary Dynamics and Trends of Exfoliative Cytology."

Each year, the papers given at the Symposium are published in the journal. *Texas Reports on Biology and Medicine*, through the courtesy and co-operation of Dr. Chauncey D. Leake, Director of The University of Texas Medical Branch and editor of the journal. Sincere appreciation is given Dr. Leake and the associate members of his staff who make the publication of the Symposium papers possible.

THE INFLUENCE OF HORMONES UPON SOME CONSTITUENTS OF CONNECTIVE TISSUE IN PROSTATIC CANCER

JOHN A. ARCADI*

"If, by some magic solution, one could dissolve all the connective tissue of the body, all that would remain would be a mass of slimy epithelium, quivering muscle, and frustrated nerve cells." In such a way the importance of connective tissue was pointed out to this speaker some ten years ago by Doctor Marion Hines. Indeed, even now I regard it in just such a light.

Generally speaking, the connective tissue in all organs of the body is similar. It has both a fibrillar and a nonfibrillar component. The fibrillar component consists of collagen, reticular and elastic fibers, whereas the nonfibrillar component is the "ground substance" or intercellular cementing substance through which pass the fibers of the connective tissue. The ground substance is believed to exist in a gel-like state that may vary in its consistency (Bensley, 1934; Clark and Clark, 1933; Gersh and Catchpole, 1949). Recently it has been suggested that the carbohydrate-protein complex of the ground substance is in a polymerized form and that the consistency of this ground substance may vary with the degree of polymerization of this glycoprotein (Gersh and Catchpole, 1949). This intriguing hypothesis has been extended by several workers (Engel, 1952; Joseph, 1952) and substantiating data have been obtained. The role of the ground substance in several disease processes has been delineated, particularly in ulcerative colitis (Levine, *et al.*, 1951), allergic rhinitis (Rappaport, *et al.*, 1953), and gingivitis (Engel, *et al.*, 1950).

Indeed, changes in the state of the ground substance have been produced by hormonal manipulation in the thyroid (Gersh and Catchpole, 1949), ovary (Catchpole, *et al.*, 1950), testis (Brown,

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1949), and pubic symphysis of the guinea pig (Perl and Catchpole, 1950).

With respect to the content of connective tissue components, the prostate gland is no different from other organs in the body. Different it is, however, with respect to its dependence on the presence of a testicular substance to maintain its size and activity. John Hunter in the early part of the nineteenth century observed that the prostate was small and fibrous in a castrated bull, whereas in a normal intact animal the prostate was "large and pulpy." Other observations (White, 1895; Moore and Price, 1932; Huggins and Stevens, 1940) on the endocrine relations in prostatic growth were culminated in the work of Huggins, *et al.*, on prostatic cancer (Huggins, *et al.*, 1941, *et seq.*). This group demonstrated that castration and estrogen administration in disseminated prostatic cancer produced a marked diminution in growth of the tumor. Although great effort has been expended, little further knowledge on the *modus operandi* of this type of treatment has been obtained. It is true that orchiectomy removes prostate-stimulating testicular androgen; and estrogen, presumably through the pituitary, inhibits testicular androgen. Nuclear pyknosis, loss of cell structure, and "fibrosis" have been frequently seen to occur after "antiandrogen" therapy (Huggins and Stevens, 1940; Schenken, *et al.*, 1942; Rezek, *et al.*, 1951; Nicol, *et al.*, 1952). But for the most part the local mechanism through which these changes are mediated has been ignored. It occurred to the writer that little consideration had been given to the possibility that an alteration of the consistency of the connective ground substance might play an important role in the changes produced in prostatic cancer by orchiectomy and/or estrogen administration.

I wish to present some of our observations relative to the above hypothesis.

Materials and Methods. Prostatic tissue was obtained by perineal prostatic punch biopsy, by open perineal prostatic biopsy, or by radical perineal prostatectomy. Nine patients were studied. In six, tissue was obtained before estrogen had been administered or orchiectomy performed. In four of these six patients estrogen was subsequently given (1 mg. t.i.d.) or orchiectomy performed. Biopsies were performed one to six weeks after therapy was

started. In three patients estrogen had been given one to three weeks before the tissue was obtained.

On obtaining the tissue in the operating room it was immediately frozen in isopentane cooled to about —155° C. in liquid nitrogen. The tissues were then transported, still frozen, to the laboratory where they were dehydrated *in vacuo* at —30° C. for 48–72 hours in our own simplified freezing-drying apparatus (Arcadi and Tesar, 1954). When dry the tissue was infiltrated *in vacuo* in 52° C. paraffin and embedded in similar material.

This method of fixation is superior to the more commonly used techniques, because it avoids denaturation of proteins, reduces diffusion of cellular materials to a minimum, and eliminates most postmortem changes.

The tissue was sectioned and mounted flat on slides by finger pressure and slight heat. Paraffin was removed by immersion in petroleum ether. The sections were denatured in absolute alcohol overnight, either directly, or after the application of extractives. They were then stained with the periodic acid-leucofuchsin technique of McManus (1946, 1948) and Hotchkiss (1948). This staining reaction visualizes polysaccharides and protein-bound or lipid-bound reactive compounds such as glycogen, glycoprotein and glycolipid. The reaction depends on the oxidation of vicinal hydroxyl or hydoxyl-amino groups to aldehyde, which reacts with the leucofuchsin to form a rose-purple colored addition product.

The total reactive material in a section is visualized by denaturing in absolute alcohol, and staining in periodic acid-leucofuchsin.

Since our study was confined to changes in the glycoprotein of the ground substance and basement membrane, the possibility of a reactive glycolipid or glycogen in these regions had to be eliminated. Sections were incubated in a chloroform-methanol solution, 1:1, for sixteen hours at 60° C., to remove any reactive lipid. Other sections were denatured in absolute alcohol overnight and then incubated at 37° C. with saliva at pH 7.0 for from one to four hours, to remove glycogen. There was no decrease in the staining reaction after either procedure indicating that there is little or no glycogen or glycolipid in the interacinar spaces and along the basement membrane. From these and previous studies

(Gersh and Catchpole, 1949; Catchpole, 1950a; Arcadi, 1952), we concluded that the stainable material in the ground substance and basement membrane is a glycoprotein or carbohydrate-containing protein rather than glycogen or glycolipid.

The stainable glycoprotein of the ground substance in various tissues investigated by others (Gersh and Catchpole, 1949; Catchpole, 1950b) is water-insoluble under normal conditions. This substance, however, can be removed from the section by the prolonged action of the enzyme present in the toxin of *Clostridium welchii* (Collagenase). Also, in the region of tissue injury and areas of tumor growth, the stainable glycoprotein becomes soluble in buffer at pH 7.0. The prostate gland, however, has not heretofore been investigated in regard to the state of the ground substance.

To evaluate the degree of polymerization of ground substance, sections of prostate were covered with buffer pH 7.0 for up to sixty hours at 37° C., denatured overnight in absolute alcohol, and stained by the Hotchkiss method. Untreated sections were stained directly, to reveal the total insoluble reactive material in the section. Other sections were treated for up to sixty hours at 37° C. with a solution of Cl. welchii toxin diluted 1:5 with buffer pH 7.0, or with a solution of hyaluronidase in buffer pH 7.0 (1 mg./cc.).

Observations. In all tissue studied there appeared to be two points of difference between the ground substance of untreated prostatic cancer and that of prostatic cancer treated by estrogen or orchiectomy. The first point is the almost complete absence of a distinct periodic acidleucofuchsin reactive basement membrane around the acini of untreated carcinoma (Fig. I) as opposed to the distinct, well delineated basement membrane in treated prostatic carcinoma (Fig. II). The second difference is in the response of the interacinar ground substance and basement membrane to enzyme and buffer activity. The untreated prostatic carcinoma has a ground substance that is partially extracted with buffer at pH. 7.0 (Fig. III) and completely removed by *Clostridium welchii* toxin, both acting for twenty-four hours at 37° C. The treated prostatic carcinoma has a ground substance that is resistant to the action of buffer and of *Clostridium welchii* toxin after incubation for twenty-four hours at 37° C. (Fig. IV). In

these studies hyaluronidase had no visible effect on the glycoprotein of the ground substance of either the treated or untreated prostatic carcinomas. No difference was noted between the ground substance of prostates treated by orchiectomy or estrogen administration.

FIGURE I

Discussion. Whether the effect of estrogen administration is a direct one, or is mediated through inhibition of pituitary gonadotrophic substances, cannot be clarified from our observations. In this particular work we are primarily concerned with the response of the ground substance of the prostate to estrogen treatment and orchiectomy and not with the general systemic hormonal mechanisms involved in such treatment.

Other investigators have shown that the connective tissue of several organs appears very clearly to be under hormonal control, *e.g.*, the ovary, the testis, the pubic symphysis, the nasal mucous membrane, the thyroid, and the colon. The observations

we have presented suggest that prostatic connective tissue is likewise under hormonal control.

Our data show that in untreated prostatic carcinoma the basement membrane is much more soluble than after orchiectomy or estrogen administration. This change may be interpreted as one

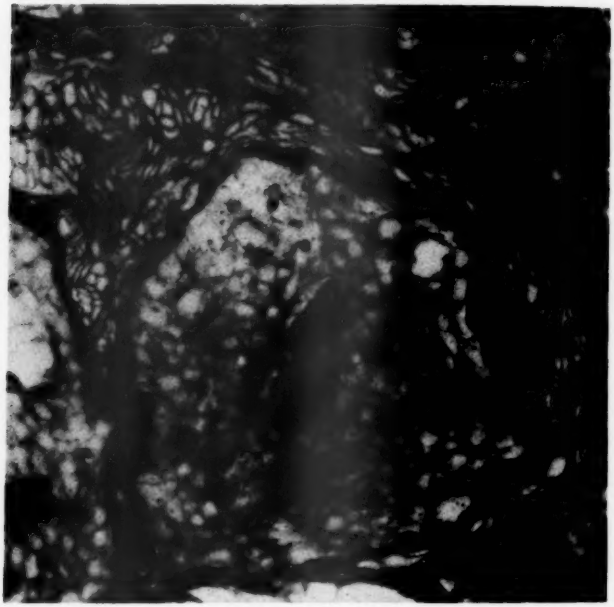

FIGURE II

in which the molecular aggregates of the glycoprotein component of the basement membrane become smaller and hence more soluble. Using this hypothesis, estrogen and orchiectomy appear to reverse or inhibit this molecular breakdown or disaggregation. This hormonally induced alteration of the ground substance would increase viscosity and resistance of the ground substance colloid.

In considering the relationship between the physical state of the ground substance and the acinar cells of the prostate, at least two assumptions are, and must be made: (1) that the more *in-*

soluble ground substance is "tougher" than the more *soluble* ground substance, and (2) that the "toughness" of the ground substance is the chief factor which determines how much resistance this material offers to the growth of cells or to their spread as in cancer. With these assumptions, it is possible to

FIGURE III

conclude that the observed changes in the basement membrane described above may contribute significantly to the mechanism of the inhibitory effect of estrogens and orchiectomy on prostatic growth.

Earlier we pointed out that the ground substance in untreated prostatic carcinoma is extremely soluble in buffer solution pH 7.0. We suggested that the molecular aggregates of the glycoprotein component of the ground substance become smaller and hence more soluble. One might then expect that if the breakdown of glycoprotein molecules proceeds far enough, the material might pass into the lymph and vascular systems and produce an

elevation of the serum glycoproteins. It is of interest to note that other workers have demonstrated an elevation of the carbohydrate-containing proteins of serum in patients with carcinoma (Seibert, 1947; Winzler and Smyth, 1948; Shetlar, 1950). The values of serum glycoproteins in prostatic carcinoma has not been

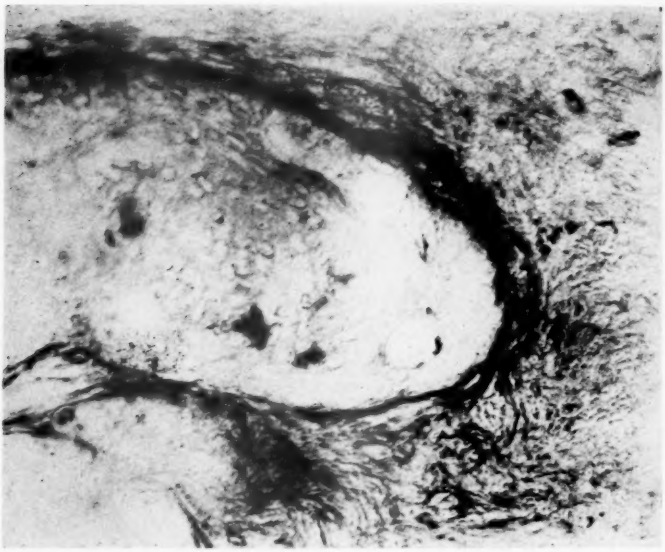

FIGURE IV

investigated, although a few isolated reports are seen in the literature. In an attempt to corroborate our histochemical observations, and perhaps to obtain substantiating evidence for our thesis concerning the action of orchiectomy and/or stilbestrol on prostatic cancer, we have investigated the serum glycoproteins in patients with this disease.

Materials and Methods. Two groups of patients were studied. Group I consisted of nine individuals without clinical, radiological, or biochemical evidence of prostatic cancer. This was the control group. Group II consisted of seven patients with disseminated prostatic carcinoma. Six had radiologically demonstrable metastases and elevated (slightly to greatly) serum acid

phosphatase. One patient had multiple cutaneous metastases from prostatic carcinoma, and a normal serum acid phosphatase. Group II was treated after baseline studies were made by bilateral orchiectomy and/or stilbestrol administered in a dose of 3 mg./day.

Blood was drawn with the patient in the fasting state, at the same hour every morning. The serum was separated from the cells, quickly frozen and stored at 0° C. until the determination was carried out.

Carbohydrate-containing proteins, or glycoproteins, were determined by a modified Winzler method (Winzler, *et al.*, 1948). In this simple technique proteins other than those containing carbohydrates are precipitated with perchoric acid. The filtrate is then treated with 5 per cent phosphotungstic acid in 2N HCl solution to precipitate the glycoproteins. The carbohydrate moiety of the glycoprotein is then determined using the orcinol reaction with a galactose-mannose standard. The intensity of the color reaction is then measured in a photo-electric colorimeter.

Results

In the control group (Group I) the values of serum glycoproteins ranged from 9.0 to 17.2 mg. per cent with *an average of 13.2 mg. per cent*. This group consisted of men in the "prostate age," with the exception of two healthy interns with small prostates.

In patients with disseminated prostatic cancer, Group II, serum glycoproteins, before treatment, ranged from a patient average (that is, an average of several days determinations on the same patient) of 23.3 to 55.6 mg. per cent. An overall *average of 37.5 mg. per cent* of carbohydrate was found in this group. The average amount of serum glycoproteins in patients with disseminated prostatic cancer was almost *three times* the average in the normal control group.

From these data we observe that our patients with disseminated untreated prostatic cancer have serum glycoprotein levels of 20 mg. per cent or more; those without disseminated prostatic cancer have a serum glycoprotein level of under 20 mg. per cent.

Five patients were studied as to the response of their serum glycoproteins to bilateral orchiestomy and/or stilbestrol administration. Four out of the five patients had a good clinical response to the therapy used. The fifth patient (with multiple cutaneous metastases, and a normal acid phosphatase) had little clinical response to the therapy.

In all five patients a decline in the serum glycoprotein levels was noted one to seven days after the therapy was begun. The graphed result in one patient is shown in figure V.

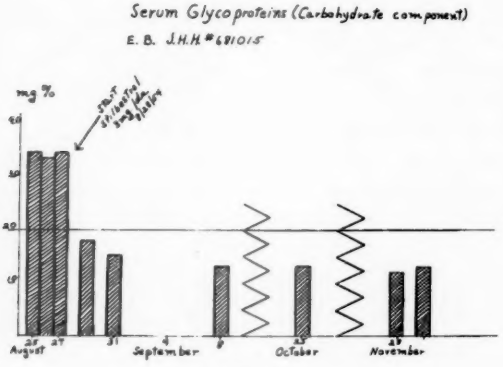

FIGURE V

In four patients, the glycoproteins of the serum dropped to levels below 20 mg. per cent after treatment was instituted. They remained at this level for from three to six months. In one patient (A. S.) serum glycoproteins increased three months after bilateral orchiectomy although he was clinically in good condition. Now, six months after orchiectomy he is having back pain from his metastases, and the serum glycoproteins have risen to about 40 mg. per cent.

Discussion. The observations that the serum glycoproteins are elevated in disseminated prostatic cancer, and that the serum levels fall after orchiectomy and/or stilbestrol administration are in agreement with the histochemical observations and hypothesis presented in the first section of this paper.

These observations on the serum glycoproteins tend to support the thesis that in untreated prostatic carcinoma the molecular

aggregates of glycoprotein of the ground substance are more soluble and tend to pass into the vascular system; after "antiandrogen" therapy the glycoprotein is less soluble because the molecule is larger or more polymerized and does not readily pass into the circulation.

The observations presented here indicate clearly that the nonfibrillar component of the connective tissue of the prostate is influenced by androgenic and estrogenic hormones, and that the changes produced in the ground substance influence the growth of prostatic carcinoma.

Acknowledgment

The author wishes to express his thanks to Mrs. Josephine Miller, Miss Alice Barnhart, and Mrs. Joyce Eversole for technical assistance in part of the work reported.

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THE HISTOCHEMISTRY OF NORMAL AND ABNORMAL GROWTH IN THE HUMAN ENDOMETRIUM*

WILLIAM B. ATKINSON

Since the discovery some 30 years ago that growth and differentiation of the various tissues of the female reproductive tract are controlled to a great extent by the endocrine secretions of the ovaries, much information concerning these processes has been gained through the use of the traditional staining methods of histology and cytology. With the development of new histochemical technics by Lison, Gomori, Lillie, Seligman and many others during the past decade, it has become possible to bring new light to bear on morphologic and physiologic problems through the study of the chemical constituents of tissues microscopically. The present paper is intended to be a review of the present status of the microscopic histochemistry of growth and differentiation of the human endometrium. It will treat the available material selectively in that it will be concerned primarily with those studies which have been complete in themselves or which have attempted to correlate histochemical findings with other aspects of reproductive physiology or pathology.[1]

Polysaccharides

Glycogen. The microscopic study of glycogen (and also of lipids) in the endometrium proceeded more or less simultaneously with the elucidation of the histologic changes which occur during the menstrual cycle because empirical but none-the-less highly selective staining methods for these substances had been

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[1] Since the descriptions of the various tissue components discussed in this review have been abstracted both from the published observations of other investigators and from the personal experience of the author, it has not been feasible to annotate the text with specific literature citations. Instead, a classified bibliography is provided at the end of the review.

developed by the beginning of the present century. Although the earlier observations on glycogen were made on sections of endometrium stained with Best's carmine solution, the results have been confirmed in all essential points in more recent studies utilizing chemically more specific technics.

During the menstrual cycle glycogen is found principally in the several tissues comprising the parenchyma of the endometrium functionalis. The material is seen as precipitated granules of varying size in the cytoplasm of the surface and glandular epithelium and in the cells of the stroma. It appears in the latter portion of the proliferative phase in relatively small quantities, increases markedly during the secretory phase and diminishes with the onset of the premenstrual phase. In the glands glycogen is first present as finely dispersed particles in the basal cytoplasm of the epithelial cells. Within a day or two after ovulation the granules coalesce to form one or two large masses, the "basal vacuoles" seen in routine preparations. The glycogen then moves rapidly toward the apices of the cells and is extruded into the lumena of the glands throughout the active secretory phase of the cycle.

Generally only traces of glycogen are found in cystic glands, even when the latter are found in the same endometrium with normal glands containing large amounts of the material. In adenocarcinoma the amount and distribution of glycogen in the neoplastic cells is variable and does not appear to be correlated with the degree of differentiation of the tumor.

Mucopolysaccharides and mucoproteins. These substances are grouped together here because they have not usually been distinguished from one another in past histochemical studies. Compared with glycogen, relatively little is known regarding the other carbohydrates in the endometrium. Our knowledge of the acid mucopolysaccharides is derived from their strong metachromatic staining with basic dyes such as toluidin blue and thionin. Although the descriptions of various authors vary somewhat in detail, due probably to differences in staining procedures, it is apparent that metachromasia is present in the intercellular ground substance of the stroma and probably in the cytoplasm of the stromal cells themselves during the proliferative phase, but disappears during the secretory phase. Whereas the secretions

in the lumena of the glands are metachromatic throughout the proliferative and secretory phases, the cytoplasm of the cells remains orthochromatic.

In a study not yet completed, the present writer has found that, unlike the metachromatic polysaccharides, those stainable with leucofuchsin after periodic acid oxidation (applied after the removal of glycogen by diastase digestion of the sections) increase in both the glands and stroma during the latter half of the cycle. The secretions of cystic glands are generally both metachromatic and periodic acid-leucofuchsin positive. A few of the adenocarcinomas seen to date have shown large amounts of mucopolysaccharide in the lumena of the glandular formations, but this is not the usual situation.

Lipids

The only definitive microscopic studies on endometrial lipids appear to have been limited to tissues stained with oil soluble substances such as the sudan dyes. Sporadic observations on specific types of lipids such as cholesterol and plasmals have been reported but the information gleaned from such sources is insufficient to establish the relationship of these substances to the cyclic activities of the endometrium. Since the sudan-type dyes are freely soluble in most fluid lipids, with the possible exception of pure phospholipids, their use sheds little or no light on the types of lipids present.

Our knowledge of endometrial lipids is further limited by the fact that, whereas a considerable number of studies have appeared, the descriptions of different authors conflict over the details concerning the amount and distribution of the sudanophilic lipids in the various phases of the cycle. It does seem to be generally agreed, however, that both stromal and glandular lipids are minimal during the early proliferative phase and increase considerably after ovulation. In the experience of the present writer sudanophilic lipids are most consistently present in the stromal cells in the most superficial ¼ to ⅓ of the endometrium during the late secretory phase. Scattered droplets appear in the functional portions of the glands earlier in the secretory phase and disappear before the premenstrual phase.

In the small series of carcinomas which we have examined to

date (8 specimens) it has been interesting to note that whereas lipid has been minimal in the neoplastic epithelial cells, the stroma of the tumors has consistently contained considerable amounts. The stromal cells appear to be normal morphologically, so for the present at least we do not believe that the presence of lipid in these cells is indicative merely of tumor degeneration.

Nuecleic Acids

Desoxyribonucleic Acid. Despite the fact that quantitative analysis of the endometrium has revealed that there is a considerable cyclic variation in desoxyribonucleic acid content, it has not been verified histochemically. The reason for this is that although desoxyribonucleic acid is easily demonstrated qualitatively in the nuclei of the various component tissues of the endometrium, its quantitation even by microphotometry is difficult and has not been attempted to date.

Ribonucleic Acid. The cytoplasmic ribonucleic acids, particularly those in the endometrial glands, have been studied rather intensively by comparing the basophilic staining of otherwise untreated tissue sections with that of parallel sections treated with ribonuclease. It has been found that during the proliferative phase the cytoplasm of the gland cells is filled with ribonucleic acid-containing basophilic granules and that occasional granules are present in the scanty lumenal secretion. During the early secretory phase the basophilic material in the infranuclear cytoplasm is displaced apically by the large glycogen granules which appear at this time, and the amount of basophilic material in the lumenal secretion increases during this phase of the cycle. During the latter half of the secretory phase there is a marked reduction in cytoplasmic basophilia, although there is a concomitant increase in ribonuclease-resistant basophilia in the glandular secretions due to the increase in acid mucopolysaccharides.

In the large majority of hyperplastic glands, both cystic and adenomatous, and in carcinomatous glands moderate to large amounts of ribonucleic acid are consistently present.

Alkaline Phosphatase

Of the several recently developed methods for the histochemical demonstration of enzymes in tissue sections only Gomori's

alkaline phosphatase technic has been widely applied in the study of normal and pathologic endometria. It should be noted that the earlier descriptions of alkaline phosphatase in nuclei should be discounted in view of recent experiments which have shown that the enzyme is present only in the cytoplasm. The Gomori technic has been recently revised to eliminate the artifactitious staining of nuclei.

Cyclic changes in alkaline phosphatase are found in the surface epithelium and in the functional portions of the endometrial glands. Little or no activity is present in the stroma except in the endothelium of the small blood vessels. During the proliferative and early secretory phases marked enzymatic activity is present in the supranuclear cytoplasm, especially at the free borders of the cells, and in the lumenal secretions. By the late secretory phase there is a sharp reduction in phosphatase activity in both the surface and glandular epithelium, and by the onset of the premenstrual phase it has virtually disappeared.

In endometrial hyperplasia and in carcinoma, as in the normal endometrium, alkaline phosphatase activity is found only in the epithelial tissues. There is a much greater variation in activity from one area to another, but the variations in phosphatase do not appear to be related to morphologic variations in gland structure. However, in the series of carcinomas studied there was a distinct inverse relationship between the over-all enzyme activity and the degree of tumor differentiation.

Discussion

It is readily apparent that the histochemical observations reviewed above present a broad spectrum of biological interest ranging from their bearing on tissue metabolism in general to their possible significance in the endometrium with respect to the physiology of reproduction in particular. Furthermore, interest in these data has ranged from the purely "academic" to their practical use in clinical gynecology.

With reference to the processes of growth and differentiation in the endometrium we may draw several general conclusions concerning the significance of our present histochemical knowledge:

(1) Regardless of the organ or tissue investigated, histochemical information is invaluable in supplementing the quantitative data of the biochemist by determining the morphologic sites in which particular metabolic processes occur. The localization of glycogen in the endometrial glands and lining epithelium is an excellent example. This is not only of direct interest in defining the sites of glycogen accumulation as measured quantitatively, but is useful in determining indirectly the sites of other chemical constitutents of the endometrium. For example, biochemical analysis has revealed that there is considerable phosphorylase activity in the endometrium and that it increases about three-fold from the proliferative to the secretory phase. Since glycogen is involved either as a substrate for or as an end product of phosphorylase activity, it is reasonable to assume that this enzyme must be located in the epithelial components of the endometrium. Other examples might be cited, but this one is sufficient to illustrate the general point we wish to make.

(2) The morphologic localization of the chemical constituents of tissues may also serve to elucidate their physiologic functions. In the cases of glycogen and visible lipid it seems generally agreed that these substances represent materials stored for future use. Their production and secretion by the endometrial glands must then be associated with providing nutrients for the fertilized ovum while it lies free in the uterine lumen and during the initial stages of implantation.

The functions of ribonucleic acid and alkaline phosphatase are also speculative, although we may deduce several possibilities by comparing their biochemical potentialities with the metabolic processes known to occur in these tissues. Ribonucleic acid has been shown to play an essential role in the synthesis of protein, and it has been suggested that the nucleic acid content of the cytoplasm is therefore an index of the rate of protein synthesis by the cell. It is not surprising, therefore, to find the high content of this substance in the rapidly growing endometrial tissues during the proliferative phase of the menstrual cycle. Similarly, we may correlate the presence of nucleic acid in the apices of the gland cells during the active secretory phase with the synthesis of the protein components of the secretion. In the case of alkaline phosphatase, this enzyme has been implicated in the synthesis of structural proteins and in the phosphorylation and phosphorolysis of a number of substances concerned in the intermediary metabolism of carbohydrates and lipids, and of the nucleic acids themselves. In the cyclic endometrium, therefore, alkaline phosphatase may be involved in the metabolism of the above substances either acting on them directly as substrates or end-products or indirectly by providing phosphate ions from other substances.

(3) With regard to the significance of the histochemical observations on neoplastic growth of the endometrium, the findings seem to uphold the general viewpoint that the metabolic processes in tumors differ quantitatively rather than qualitatively from those in the tissues of origin.

If ribonucleic acid content is truly an index of protein synthesis, we might well expect neoplastic tissues to be rich in this substance, as is the case. The findings concerning alkaline phosphatase, on the other hand, are more difficult to interpret. The independence of histologic and enzymatic variation from one area of a tumor to another is enigmatic. The decreased phosphatase activity in poorly organized tumors and its virtual absence in anaplastic tumors, however, almost certainly indicates that the enzyme is not essential to tumor growth.

If we conceive of glycogen and visible lipid as being primarily storage products in normal endometrium, their appearance in neoplasia would indicate that the metabolic mechanisms concerned in their synthesis remain intact in a considerable percentage of endometrial tumors. It is interesting, but at present unenlightening, to note that the morphologic differentiation of endometrial tumors appears to be unrelated to their capacity to synthesize and store glycogen. The significance of the greater predilection of tumors to store lipid in the stromal rather than the epithelial elements is likewise obscure.

Whatever else might be said concerning our present biochemical and histochemical knowledge of the endometrium, we can heartily agree with the appraisal of Dr. S. R. M. Reynolds in his recent extensive monograph *The Physiology of the Uterus* (p. 417) "Considering the fact that numerous constituents of the uterus have been investigated by biochemists for over a quarter of a century, with the establishment of many data, it is surprising that so little is known today concerning their significance. With much information available concerning the hormonal and physical factors involved in uterine growth and activity this would seem to be a promising field for research."

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEIN*

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To histochemists and morphologists, proteins are not only ubiquitous but their separate demonstration has always been elusive. The interest of the histochemist in proteins is natural; they have a unique significance in biology, in that they are an indispensable substrate of cells. Wherever the phenomena of growth and reproduction occur, proteins are primarily involved. Some proteins form inert substances, in tissues such as hair or horn; contractile substances, such as muscle protein; biologically-active substances such as hormones, or enzymes. These proteins, which are the products and constituents of cells, occur in complex mixtures, not only with each other, but with other substances. They exist in various solid and dissolved states. Even to the physical chemist or the biochemist, they are as yet exceedingly difficult to separate and identify completely. The histochemist is even more limited since he cannot treat his protein harshly. Chemically he is confined to a two-phase system. His protein, denatured and precipitated, therefore abnormal from the start, is in what is known as a tissue section. His reagents are in solution. The interphase reactions that go on between the reagents and the protein are little understood. The task of the present-day histochemists seems to be to investigate the constitution and architecture of various cells and cell structures, and to discoved, if possible, the way in which structure conditions biological processes, especially biochemical or physiological events.

The subject of the histochemistry of proteins may be approached by a discussion of the sulfhydryl groups of cysteine, and the disulfide groups of cystine, two amino acids present in

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many proteins. By demonstrating these groups histochemically, the possibility is provided for revealing the sites of proteins in general, and of certain proteins in particular, by means of a component part of their amino acid composition and ultimately to study morphologically the changes in concentration of a functionally important group in various physiological states.

Histochemical Methods for Sulfhydryls and Disulfides. Although methods for the histochemical demonstration of sulfhydryl groups have been known since 1902, and there are a number of methods available, these have been reviewed elsewhere (Barrnett and Seligman, 1954). In this communication, reference will be made to the seven histochemical methods developed in collaboration with Dr. A. M. Seligman within the past few years. In order to improve the sensitivity of sulfhydryl histochemistry by increasing the color value of the final compound, and by increasing the specificity of the reactions for sulfhydryls, a new reagent, 2,2′ – dihydroxy–6,6′ – dinaphthyl disulfide (DDD), was developed which contained a disulfide linkage, the specific oxidating group, and a naphthol moiety for coupling to form an azo dye (Barrnett and Seligman, 1952). The rationale for using this compound depends upon the specific oxidation of sulfhydryls, and no other groups in protein, by disulfides at an alkaline pH. The reaction between protein-bound sulfhydryl and the reagent is reversible, and readily influenced by mass action. However, the reaction is not merely oxidation and reduction with transfer of electrons, but the chromogenic moiety, naphthol, is transferred to protein and becomes part of the oxidation product from which a colored compound is produced. The azo dye which is produced by coupling with tetrazotized diorthoanisidine is either red (monocoupling), or blue (dicoupling). Monocoupling diazonium salts may be used to produce other azo dyes, each having one color of varying intensity. The reaction between DDD and protein-bound sulfhydryl groups is specific and is the proof of the specificity dealt with extensively elsewhere (Barrnett, 1953; Barnett and Seligman, 1954a).

Since native proteins and enzymes usually assume a rigid steric configuration, the sulfhydryl groups within them may exhibit different degrees of reactivity, owing to the different orientation of the sulfhydryl groups within the protein. Such differences

may conceivably be demonstrated by various reagents for detecting sulfhydryl groups. Therefore, the development of other methods for comparison with DDD would seem worthwhile. Since it is well established that N-ethylmaleimide reacts with sulfhydryl groups specifically and rapidly (Friedmann, Marrian and Simon-Reuss, 1949), the usefulness of such a reagent for histochemistry was explored, and a number of N-(hydroxy-substituted-1-naphthyl) maleimides and N-(hydroxy-substituted-1-naphthyl) isomaleimides were prepared. These compounds, after reaction with protein-bound sulfhydryl groups, could be made to couple *in situ* with a suitable diazonium salt to form a protein-bound azo dye. During these studies, 4-naphthoquinone monobenzine sulfonimide was also prepared and found to react with sulfhydryl groups (Seligman, Tsou, and Barrnett, 1954; Tsou, Barrnett, and Seligman, 1955; Barrnett, Tsou, and Seligman, in preparation). In addition, a mercaptide-forming agent, 4-(N-hydroxy-1-naphthyl mercuric acetate, and an alkalating agent, 4-(N-iodoacetyl) amino 1-naphthol, were prepared, and histochemical methods were developed with these reagents and suitable diazonium salts. Protein-bound sulfhydryl groups reacted with these reagents, but in addition non-specific reactions were noted (Barrnett, Tsou, and Seligman, 1955).

In addition to sulfhydryls, it would be important to demonstrate disulfides as well. Not only is there an oxidation reduction system formed by these two compounds in many tissues, but some proteins are rich in disulfides only, and these could possibly be demonstrated by a histochemical method. For the demonstration of sulfhydryls and disulfides together, two parallel sections are needed; one stained for sulfhydryl, and the other for sulfhydryl and disulfide after reduction of the disulfide by reducing agents such as thioglycolate. The sites of disulfide may be inferred from the differences in staining produced by the two methods. For disulfides alone, sulfyhydryls are blocked, disulfides are reduced to sulfhydryls and stained. Any one of the aforementioned reagents may be used, although in the majority of our experiments we have usually used DDD. In addition, tetrazonium salts in a strong alkaline solution, with or without potassium cyanide, will stain both sulfydryls and disulfides. These methods for disulfides

alone or for both sulfhydryls and disulfides are described elsewhere in detail (Barrnett and Seligman, 1954a).

Distribution of Sulfhydryl and Disulfide Groups. Sulfhydryl groups are widely distributed in vertebrate tissues. In adult animals the cytoplasm of cells almost always contains more sulfhydryl than the nuclei. All epithelia contain sulfhydryl, although the quantity varied among the epithelia of different tissues. Muscle fibers, some of the elements of the nervous system and various blood and connective tissue cells were also reactive for sulfhydryls. The only tissues which were clearly negative for sulfhydryl were collagenous fibers, the matrix of bone and tracheal hyaline cartilage, mucus, elastic tissue and parts of some fully keratinized tissues (Barrnett, 1953).

Most of the sites which were positive for sulfhydryl also contained disulfide. This is not an unexpected result, since, sulfhydryls and disulfides provide an intracellular oxidation-reduction system in many tissues. The reversibility of the system, $2SH - 2\,\bar{e} \longleftrightarrow S\text{-}S + 2\,H^+$, and the specificity of the reactants (SH and S-S) for each other (Barron, 1951), suggest that in many instances they would be found at the same sites. However, frequently a predominance of either sulfhydryl or disulfide groups was found in some cells, indicating that the equilibrium was shifted far to one side or another, or that certain cells contained a predominance of sulfhydryl or disulfide-containing prote'n. A number of these special sites will be briefly reviewed.

Keratinous tissues. The keratins are proteins which probably contribute to the architecture of the cells of the epidermis and of the cells of epidermally-derived tissues by virtue of their fibrous nature. Previous observations with the present methods (Barrnett, 1953; Barrnett and Seligman, 1954a; and Barrnett and Sognnaes, 1955) on epithelium and epithelial derivatives, which are known to contain keratins, conflict with the generally accepted accounts of the histochemical distribution of sulfhydryl groups in keratins (Giroud and Bulliard, 1933; Giroud and Leblond, 1951; Chévremont and Frederic, 1943). We have found, on investigation of keratinous tissues of various fish, reptiles, birds and mammals that all keratinized tissues, excepting some animal hairs, contain free sulfhydryls, and that none of these groups are oxidized to disulfides during the process of kera-

tinization. Disulfides are not usually restricted to the fully keratinized tissues, but disulfides as well as sulfhydryls are present in prekeratinous areas. A "keratogenous zone" rich in sulfhydryls and supposedly only occurring in hard keratins (Giroud and Leblond, 1951) is absent in some hard and present in some soft varieties. Although soft keratin (epidermis) contains a transitional zone between the Malpighian and the keratinized layers that stains poorly in histological preparations, no such zone can be demonstrated in sections stained histochemically for sulfhydryl and disulfide. Keratohyaline and trichohyaline granules, which have been said to play a fundamental role in the process of keratinization (Chévremont and Frederic, 1943; Branca, 1911), contain no sulfhydryls or disulfides. This finding does not support the concept that these granules are keratin precursors. In hair, frequently used as a model for hard keratin, the hair bulb and the proximal one third of the developing hair shaft are rich in sulfhydryl groups, whereas, the remainder of the hair shaft which is fully keratinized is rich in disulfides and negative for sulfhydryls. The distribution of these groups is quite different in claws, nails, horns, hoofs, feathers, spurs, beaks, scales and horny teeth in which both sulfhydryl and disulfide positive protein is present in the fully-formed keratins.

Neurohypophysis. This study concerning neurohypophyseal hormones and some of the following studies illustrate experiments in which specific proteins known to contain disulfide groups may be demonstrated histochemically. In these experiments, certain criteria must be satisfied. These are: that the hormone or its precursor contains disulfides; that the disulfide groups can be demonstrated by a specific method in the cells of the tissue known to contain the hormone; that available extracts of the hormone could be shown to be rendered insoluble by the same fixation as was applied to tissue, and to stain by the same histochemical method for disulfides *in vitro;* that physiological experiments would reveal alteration in the staining reaction in a predictable manner as the hormone is stored or released. In the studies on the neurohypophysis (Barrnett, 1954), a method was found to precipitate neurohypophyseal hormones by *in vitro* experiments so that the precipitates would not dissolve in any of the histological procedures necessary for the preparation of tissue

sections, or in any of the histochemical procedures necessary to stain disulfides. When the result of these *in vitro* experiments were applied to sections of neurohypophysis of various normal animals, disulfide-positive, colloid-like droplets which appeared to be the same as neurosecretory material, which is stained selectively with chrome-alum hematoxylin (Scharrer and Scharrer, 1954), was preserved. Similar positive disulfide material was present in the infundibular stalk, the hypothalamo-hypophyseal fibers, and the supraoptic neurones of normal animals. Neurohypophysis, fixed in reagents which dissolved the neurohypophyseal hormones showed absence of the disulfide-positive material. The disulfide-positive material was completely depleted from rats which had been severely dehydrated, and it reaccumulated upon rehydration. It was also depleted within five minutes after an acute painful stress. Excessive hydration produced by feeding experimental animals a diet of dilute milk, caused a moderate depletion or a moderately excessive accumulation of the disulfide-positive material. After section of the infundibular stalk, or after hypophysectomy, large amounts of the disulfide-positive material accumulated in the median eminence, a finding never present in normal animals. Most of the stalk-sectioned animals showed complete absence of the material in the infundibular processes. These studies suggest that the material demonstrated histochemically is closely associated with, and may actually be, neurohypophyseal hormones which are known to contain disulfide groups.

Insulin. In continuing experiments for the demonstration of individual proteins particularly rich in disulfide groups by a specific histochemical method for disulfides or of regions in tissue where such proteins were concentrated, attention was turned to insulin. Again it must be stressed that success or failure to carry out such a demonstration, would depend, in part, upon whether the solubility and extractability of insulin under the conditions of the method could be avoided. Insulin, although deficient in sulfhydryl groups, is rich in disulfides by virtue of its 12 percent cystine content, it is available in crystalline form for critical *in vitro* experiments for working out details of the histochemical procedure, and a good deal is known about the chemistry of insulin (Jensen, 1948) as well as the changes in concentration

in the pancreas under different physiological conditions (Haist, 1944). With *in vitro* experiments, a fixative was found (Romeis's fluid) which would precipitate insulin so that it was not redissolved by any of the histological procedures necessary for the preparation of tissue sections, or any of the histochemical procedures necessary for the staining of disulfides. When blocks of pancreas were fixed in this manner, and the sections stained histochemically, disulfides were revealed in the cytoplasm of beta cells of the islets of Langerhans. When fixatives which did not render insulin insoluble were used, this characteristic histochemical reaction was absent. In addition, a method was found to dissolve the insulin once it had been precipitated irreversibly for the conditions above. Sections stained for disulfide after this procedure were identical to those fixed in fixatives which did not precipitate insulin. In addition to the study of the pancreases of normal animals, experiments designed to alter the content of insulin in islet cells were conducted. These experiments form an integral part of our hypothesis and provide a means of cross-checking the variation of the intensity of the histochemical reactions, with the results of others who hypothesized a change in insulin content on the basis of selective stains for beta granules, or on the basis of insulin bioassay. In these experiments, the alteration in disulfide content of the beta cells was studied in rabbits after starvation, partial pancreatectomy, glucose infusion, and insulin, diethyldithiocarbamate or alloxan injection, and in mice, genetically obese and hyperglycemic, some of which were treated with diethyldithiocarbamate. In each of these experiments the alteration of the disulfide in beta cells was the same as the alteration of the staining of the beta granules by the aldehyde fuchsin method of Gomori (1950) which does not stain insulin *in vitro*. The decrease or increase in disulfides parallels the results of others who had performed similar experiments and determined the insulin content by bioassay. These experiments are reported in detail elsewhere (Barrnett and Seligman, 1954b; Barrnett, Marshall, and Seligman, 1955).

Adenohypophysis. In previous work (Barrnett and Seligman, 1954a; Ladman and Barrnett, 1954) it was found that acidophiles of various animals contained a significant amount of sulfhydryl and disulfide containing protein histochemically,

whereas, basophiles and chromophobes contained little or no reacting material. It is reasonable to expect disulfide-containing protein to be present in adenohypophyseal cells since it has been reported that some adenohypophyseal hormones, especially Growth, ACTH, and LTH contain disulfide (Li and Evans, 1948). To relate the presence of the disulfide-containing protein to the presence of protein hormones, two series of experiments were performed. In the first series, the adenohypophyses of rats and mice in various abnormal endocrine states were stained for sulfhydryl and disulfide (Ladman and Barrnett, 1954a). It was found that, after castration, there was a perceptible accumulation of disulfide-positive material in basophiles. The acidophile content of disulfides remained unchanged. The adenohypophyses of dwarfed mice, which contained no growth hormone, exhibited no intense reaction for disulfides in any of the cells. Within five minutes after an acute stress, the disulfide material in acidophiles of rats decreased markedly. After a chronic stress (seven to fourteen days at 5° C.) some acidophiles showed an increase in this material. The adenohypophyseal acidophiles of normal rats receiving a daily injection of cortisone or desoxycorticosterone for four to seven weeks appeared like those which had been stressed by cold. On the other hand, acidophiles of rats fed propylthiouracil for six weeks showed almost complete absence of the staining for these groups (Ladman and Barnett, 1955).

The second series of experiments was based upon the findings that the adenohypophyseal cells of the rat could be divided into those that stained with the periodic acid Schiff (PAS) method, those that stained for sulfhydryl and disulfides and cells which have little reactive material (chromophobes). In addition, the assumption was made that the PAS-positive material could be the glycoprotein hormones FSH, TSH and LH, and the disulfide positive material could be the simple proteins STH, LTH and ACTH. In order to prove this assumption, a series of experiments combining the use of differential protein solubilities, histochemical staining and bioassay was undertaken (Barrnett, Ladman, and McAllaster, 1955). The glycoprotein hormones were studied first, and a solvent was sought which would dissolve one or more of these hormones and precipitate the remainder of them as well as the simple hormones. After the action of the solvent, the

glands were refixed in sublimate-formal to irreversibly precipitate the remaining hormones for histological and histochemical study. Other glands subjected to the same extraction were refixed in absolute acetone to precipitate the remaining hormones, biologically intact. These latter adenohypophyses were then assayed in hypophysectomized immature rats to determine the relative hormone content.

More than forty solvents were tested and no solvent was found that was capable of removing one hormone alone. However, it was found that 2.5 percent trichloracetic acid removed FSH and LH completely, but did not appreciably affect the content of LH as determined by bioassay. Histochemically the adenohypophysis was devoid of PAS-positive intracellular material, except in some basophiles. The staining of acidophiles with acid dyes, and for disulfide groups was not appreciably altered. Another solvent, 50 percent pyridine, removed all the glycoprotein hormones as proven by bioassay. Histochemically the PAS-positive material was extracted from all of the basophiles (Barrnett, Ladman, McAllister and Purvez, 1955) Similar studies on the localization of the simple protein hormones of the adenohypophysis have been completed and will be reported elsewhere (Barnett, Siperstein, Josimovich, McAllister, and Folsom, in preparation) as well as the sigle extraction of either FSH or TSH.

Rapidly growing tissues. It is well known that sulfhydryl groups are important in cell growth and mitosis. The work in this field has been recently reviewed by Mazia (1954). Our studies, still in progress, started with a survey of the histochemical distribution of sulfhydryl groups in various tumors (Reiner and Barrnett, unpublished results). Although more than eighty human tumors have been collected, no single type of tumor is represented by more than a few samples. Benign tumors, generally contained less sulfhydroyl than malignant ones, and of the malignant tumors, those of epithelial origin usually contained more sulfhydryl than the others. No definite positive correlation could be drawn between the amount of sulfhydryl groups revealed histochemically and the degree of malignancy anticipated from histopathologic criteria. Disregarding the differences in sulfhydrly content between tumors, one result was clear: that, in man, malignant neopplasm, the nuclei, and especially the nu-

cleoli, were more prominently stained than those of normal adult human tissues. This was emphasized since many of the tumor cells contained little cytoplasm or the cytoplasm was highly vacuolated or gave a frothy appearance. For rapidly growing normal cells, the sulfhydryl contents of regenerating livers and embryos were studied (Marshall and Barrnett, unpublished results). Contrary to the tumors, the interphase hepatic cells after partial hepatectomy did not show more prominently stained nuclei than normal cells. In addition, it was found that the cells of the regenerating liver lobule could be divided into two types on the basis of sulfhydryl content. In the vascular areas, both portal and hepatic, the cells had a cytoplasmic sulfhydryl content, higher than normal, whereas the cells of the intermediate zone of the lobule, where mitosis most frequently occurred, contained the same or less sulfhydryl than normal. The possibility of the former cells assuming a metabolic function and the latter cells a regenerative function, suggests itself. Attention was also called to the dividing cells. There was no striking difference between the sulfhydryl content of the cytoplasm of dividing and non-dividing cells. In dividing cells the mitotic apparatus, including the chromosomes, stained more intensely than the surrounding cytoplasm. This difference could be due to either a higher sulfhydryl concentration in the protein or to a higher concentration of protein, possibilities which cannot be decided on the basis of qualitative work.

Preliminary studies of the sulfhydryl content of embryonic tissues of mice showed that, in the specimens taken from the fifth to the seventh day of gestation, there were characteristically more sulfhydryl groups in the nuclei of various tissues than those seen either in later stages of development or in mature tissues, a finding similar to that in the malignant tumors.

In rapidly growing tumors, some connective tissue fibrils were sulfhydryl-positive. Normal adult connective tissue fibrils, with the exception of reticular ones, are negative. During the first ten days of the healing of surgically-induced wounds, the developing connective tissue fibrils and their surrounding matrix were sulfhydryl positive. Thereafter, they became sulfhydryl negative.

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HISTOCHEMICAL OBSERVATIONS ON CONNECTIVE TISSUES

Including a Note on the Use of Chymotrypsin as a Mucolytic Agent for Use in Gastric Lavage for Cell Studies

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In our quest for an understanding of the nature of disease both morphology and chemistry have played important roles. But neither morphology nor chemistry alone can yield us a complete understanding of the problems for which we seek an answer. The full understanding rests upon knowledge not only of the form, gross and microscopic, and the chemical composition and metabolism of cells and tissues, but upon a knowledge of how the various chemical substances are integrated to give the organism and its parts their form and function. It is the growing awareness of this which has led to the development of the branch of biology and pathology known as histochemistry. Histochemistry bridges the gap between morphology and biochemistry. It is in the midst of its earliest growing pains and perhaps we ask too much of it yet to yield us answers to the many questions for which modern medicine is avid.

In what follows I wish to discuss the general nature of the problem involved, particularly in connective tissue histochemistry, using in part some specific examples from work currently being pursued in our laboratory.

At present we have available three main types of approach to the histochemical problem: (1) Localization of substances in tissue sections; (2) Correlation of chemical substances with morphological elements; (3) Isolation of cells and subcellular particulates. Tissue culture, a specialty in itself, plays a part in this latter category. A major portion of this paper will deal with the

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first category, but time permitting I will illustrate the other categories as well.

The problem of the localization of substances in tissue sections revolves around the following points: (1) fixation, (2) specific sensitive methods of identification. These issues have been discussed in detail by others, in particular by our eminent chairman, Dr. George Gomori. One item to which insufficient attention has been given, I believe, is the fact that in tissues we are dealing not with isolated compounds, but with complex combinations of substances. This has been treated mainly as a nuisance, but is of more than pest value in the economy of life and its structure. This multiplicity and the *in situ* interactions of the substances enormously increases the complexity of the problem of the histochemist over that of the chemist who can isolate his material to determine its structure. Some of the difficulties in histochemistry which have arisen in the past have been due to the lack of overt recognition of the fact that we are dealing in tissues with a new level of organization. Perhaps those of us who attempt to deal with the problems at this level and at this time are "fools who rush in where the angels fear to tread."

The particular substances in connective tissue which have occupied much attention in recent years are the polysaccharides, and in particular that group having hexosamine as a characteristic component and an acidic moiety. These have been designated acid mucopolysaccharides (Meyer, 1945). Within this group belong hyaluronic acid, the several chondroitin sulfates and the heparins. For the demonstration of these substances several methods have been tried. These are: (1) metachromatic staining with basic dyes, in particular toluidin blue, (2) the periodic acid-schiff combination (PAS), (3) the so-called pH signature, (4) removal of stainable material by presumably specific enzymes, and (5) solubility of substances in various solvents.

The physical chemistry of the metachromatic reaction is not understood, and we shall not, because of time limitation, be able to discuss the problem. The alcohol-resistant metachromasia is given mainly, as Lison (1935) pointed out, by the large molecular sulfuric acid esters of the type of chrondroitin and heparin. In addition the carboxyl groups produced by prolonged oxida-

tion of glycogen with chromic acid will also give the characteristic color (Brigmardi, 1940). We have found that of the defined polysaccharides purified preparations of heparin and chrondroitin are metachromatic when applied in small amounts (10–25 micrograms per square cm.) to paper and stained by toluidine blue. Hyaluronic acid is not metachromatic to any significant degree in these concentrations and is only moderately basophilic. Sylven has reported similar observations (1947). One must, therefore, view critically the many interpretations which have been made of metachromasia as indicating the presence of hyaluronic acid in tissues. At the moment the appearance of metachromasia can be used as presumptive evidence of the presence of highly negatively charged, presumably sulfated, large molecular substances.

Dempsey and coworkers (1947) attempted to further define tissue polysaccharides by the use of the "pH signature." In this they assumed that by plotting the curve of binding of a basic dye at various pHs they could distinguish between polysaccharides with different acidic groups *e.g.*, carboxyl, and sulfuric. In principle this would be true if the polysaccharides in tissues were isolated substances. In point of fact they are not, and as we have shown (French and Benditt, 1953), tissue protein may compete with the basic dye for the acidic polysaccharide. The "pH signature" therefore, may be a reflection of this competition rather than of the dissociation of the acidic groups of the polysaccharide. It is possible that the "pH signature" could have application in determining the presence of an ampholyte associated with the acid muco-polysaccharide if one had another means of first demonstrating the polysaccharide.

The periodic acid-schiff method of staining certain groupings in tissues, as developed by McManus (1947) and Hotchkiss (1948), has enjoyed great popularity. It was originally assumed by some that this was a stain for mucopolysaccharides and particularly hyaluronic acid. It now seems likely that hyaluronic acid does not stain (Davies, 1952). The reason for this has recently become apparent. There are no paired hydroxyl or adjacent aminohydroxyl groups available because of the linkages within the molecule as indicated by its failure to consume periodate (Meyer, 1954). At least some chondroitins consume peri-

odic acid with production of aldehyde groups and should therefore stain. Whether or not heparin stains with PAS may depend upon its degree of sulfation (Jorpes, *et al.*, 1948) and the variable PAS positivity of mast cells has been used as presumptive evidence of a metabolic cycle for heparin in the mast cells (McManus, 1954).

If we examine the nature of the PAS stain it becomes clear that as a primary means of identifying substances it can tell us only that where staining is seen there are present compounds having the glycol or amino-hydroxyl pairing necessary for the periodate to act. In general these combinations are present in sugars and their derivatives. Positive PAS staining may indicate the presence of polysaccharides, but not necessarily, since complex lipids and proteins containing sugars may also exhibit this behavior. Finally it should be pointed out that not all of the sugars which one might expect to give the reaction do so (Jeanloz, 1950). Hence a negative reaction does not necessarily mean absence of this group of compounds. Thus the PAS stain has a broad range and a low resolving power with respect to sugar-containing moieties and other means must be used to make the resolution.

Gersh and Catchpole (1949) suggested the use of solubility in water versus alcohol as an auxiliary method for distinguishing polysaccharides and further as an indicator of the state of "polymerization" of glycoproteins. Not only does the problem of the mixtures of substances in tissues enter into this mode of approach but also the fact that solubility conditions are far different in tissue sections from those encountered by the physical chemist in the test tube. We have encountered this in the following situation: In attempting to discover the pathogenesis of the edema produced in rats by the intravenous injection of testis extract we found that the material in the edematous regions gave a positive PAS reaction. The PAS positive material was found to be soluble in water, soluble in 4 per cent formaldehyde and insoluble in alcohol at concentrations of 50 per cent or greater. This might lead one to suspect that a polysaccharide was involved. In this instance it could be demonstrated by extraction of the edematous tissues with 0.85 per cent saline and analysis for nitrogen, hexosamine and by electrophoresis that the material staining with

the PAS was plasma protein and not depolymerized polysaccharide. This fact was instrumental in our re-examining the evidence that hyaluronidase in the testis extract was responsible for the increase in capillary permeability, and eventually concluding that it was not (Benditt, *et al.*, 1951). The solubility of plasma protein in the 4 per cent formaldehyde is at first glance startling. It is not so startling when one considers the fact that we have in the tissue sections only a few micrograms of protein in a finely divided state and to this we apply from one to fifty ml. of solvent. The solubility of the many proteins in frozen dried sections is great like that of other lyophilized material. Formaldehyde requires time to react with the protein and render it insoluble. During this time the protein dissolves and disappears from the section. The example indicates the severe limitations of the solubility method when applied to tissue sections.

Removal of stainable substances from tissue sections by specific enzymes has also been used as a method of defining location. With glycogen this is practically a routine procedure. Testis extracts and bacterial filtrates having hyaluronidase activity have been used for the purpose of demonstrating hyaluronic acid (Bunting, 1950). It is now quite clear that testis hyaluronidase can split both chrondroitin sulfate and hyaluronic acid (Mathews, *et al.*, 1951). Furthermore the crude testis extracts usually available have a multiplicity of enzyme activities including some proteolytic activity (Benditt and French, 1953). Lillie (1949) suggested that malt extract had chondromucinase activity. On investigating this we were able to demonstrate that the removal of the chondroitin sulfate from cartilage by malt diastase preparations was due to a proteolytic enzyme resembling chymotrypsin (Benditt and French, 1953). This work indicates that one must use caution in interpreting the results of the enzymatic digestion. We need known specific enzymes and not mixtures to successfully employ this approach.

Another method of approach to the problem of localization of substances in tissues is that of correlation of chemical analysis of tissues with morphological analysis. Linderstrom-Lang and Holter have been proponents of this method.

An example of the use of the correlative method is the following derived from some of our recent studies on mast cells (Ben-

ditt, Bader and Lam). We have tried several ways of demonstrating histamine in tissue sections. This has several inherent difficulties. The known color reactions for this substance depend on the imidazole ring reaction with diazo compounds. The shades produced with most diazo compounds are poor and have a low intensity. Furthermore histamine is very soluble. Since these methods did not yield the desired result we resorted to correlation of the assayed histamine content of the tissues, with counts of the numbers of cells in the tissue. For the cutaneous and subcutaneous tissues of the rat the correlation was found to be linear and to extrapolate through the origin. This supports the original claim (Riley and West, 1953) of the mast cell histamine association. Other evidence leads us to believe that the histamine is probably in the mast cell. Assuming this to be so and measuring the per cent volume in one tissue occupied by the mast cells we have computed the cell concentration of histamine to be about 1 per cent, an interesting and significant quantity.

As we see this combination of techniques can be fruitful, providing we have some prior notion of what substance to correlate with which cell.

In further attempts at analysis of the mast cell granules we have used the method of particulate separation. This has been quite successful, in the case of mitochondria. With nuclei it gives more difficulty and with the mast cell granules we have thus far been able only to isolate "ghosts" of the granules. Like the other techniques it has its proper place in the total approach to cell structure. The principal drawback to this method is the uncertainty concerning what substances may have been lost or gained during the separation.

What I have said thus far was not to discourage you. It has been to point out that there is no quick or easy solution. It will take the combination of sound chemistry, sound biology, ingenuity and perseverance to solve the problems.

Finally to show that there are at least some rewards along the way, I would like to present a piece of work relating to diagnosis of carcinoma of the stomach. Dr. Cyrus Rubin, formerly of the Department of Medicine of the University of Chicago Clinics, now at the University of Washington, Seattle, has been interested in cytodiagnosis of stomach lesions. Originally he used

papain lavage as part of his routine along with the abrasive balloon. He found papain troublesome to use for three reasons: (1) the enzyme had to be activated before use, (2) the enzyme preparations are crude and not well standardized, and (3) too long exposure of the washings especially with achlorhydric stomachs led to cell digestion. You will recall that we had found that crystallized chymotrypsin at pH 5.3 has the capacity to digest the chondroitin out of cartilage. We also tried papain and found it to have a similar, though less predictable, activity. We therefore, suggested the use of the chymotrypsin in place of papain and the use of $\frac{m}{10}$ pH 5.6 sodium acetate buffer (7 mg. Chymotrypsin (Armour) in 500 ml. $\frac{m}{10}$ sodium acetate). This buffer we reasoned would take care of small amounts of acid remaining after aspiration and yet maintain a slightly acid reaction in achlorhydric stomachs. This material has proved in the hands of Dr. Rubin and others (1955) of our Department of Gastroenterology, to be an effective form of lavage. The results with the Chymotrypsin lavage are at least as good as those with the papain or with the abrasive balloon and they have now adopted this as the procedure of choice. This procedure is not magic and requires the two ingredients necessary for any successful cytologic method, namely, careful attention to collection and preparation of the material and a competent cytological observer.

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HISTOCHEMICAL STUDIES ON THE DEVELOPING CHICK EMBRYO THYROID*

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Recent work in this department (Trunnell and Wade, 1955) employing radioactive iodine and chromatography as instruments for investigation has indicated the orderly and progressive development of the thyroid hormone of the chick embryo thyroid. The developing gland has been shown to collect iodide as early as the 5th embryonic day, iodide being present at that time in higher concentration than in any other tissue. This iodide, however, is readily removed by aqueous trichloracetic acid, indicating that the mechanism necessary for conversion to iodine or for iodinating protein is not yet under way. At 8½ days the chromatograms show the first synthesis of monoiodotyrosine and by way of correlation, the PAS reaction shows the first characteristic coloring of the colloid occurring at some point in the following 12 hours. The first traces of active SS-SH groups appear also, indicating that whatever the progression of events may be, it is off to a substantial start with the appearance of the first colloid droplets. Some 18 hours after the first MIT is in evidence, diiodotyrosine appears, followed in about 12 hours by thyroxine which is present to an appreciable degree by the 10th embryonic day. The gland apparently is capable of producing MIT and DIT before the formation of colloid, whereas thyroxine does not appear until the first colloid droplets are present.

Current histochemical interest in the ubiquitous sulfhydryl groups has prompted a study of the SS-SH groups in the thyroid colloid of the chick embryo in an effort to date, if possible, the appearance of these chemical constituents essential to many vital activities. Sulfhydryl groups appear in a wide range of complexes influencing hormone synthesis and activity, in the activties of many enzymes, in the coagulation of blood, in muscle contraction and serving as linkages between proteins and prosthetic groups, (Barrnett and Seligman, 1952). Sulfhydryl enzymes, *i.e.*, en-

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zymes requiring the SH group for activity, occur in so many of the steps during carbohydrate metabolism that it may be concluded that these enzymes play an important role in the breakdown and synthesis of glycogen (Barron and Singer, 1945). To list a few: phosphorylase, carboxylase, decarboxylase and a number of the oxidases are sulfhydryl enzymes. In the metabolism of fat, pancreatic lipase, liver stearate oxidase, and B-hydroxy-butyric dehydrogenase are among the sulfhydryl enzymes and in protein metabolism sulfhydryl enzymes include transaminase, l-glutamate dehydrogenase, and monoamine oxidase (Barron and Singer, 1945). Sulfhydryl groups are necessary not only for enzymatic activity but also for stability of the protein fraction. When SH groups have been destroyed by oxidation or mercaptide formation, complete restoration of the enzyme is possible only within certain time limits. Glutathione, having a universal distribution in intracellular fluid, may have as one function the maintaining of the SH enzymes at optimal activity, since glutathione, at a ratio of 10:1 of SH inhibitor, restored activity of the enzymes. It has been shown that adenosine-triphosphatase, when inhibited by a trivalent arsenical, was reactivated by the addition of glutathione. However, a greater percentage reactivation was obtained when glutathione was added within fifteen minutes after the inhibitor than was evident when addition occurred after an hour had elapsed. Barron (1948-49) indicates 2 kinds of SH groups: first, the soluble SH groups present in the water phase of the cell which regulate respiration by the inhibition of reoxidation of the cytochrome system and second, the fixed SH groups bound to the protein component of cellular enzymes, In his work, small concentrations of SH reagents produced an increase in cellular respiration, whereas increased amounts inhibited it. This suggested to him that the lower concentrations used up the readily available SH groups and removed the brake on respiration, whereas the higher concentration interfered with the primary respiratory mechanism. Over 50 per cent of respiratory enzymes contain SH groups and Slingerland (1955) has shown that many SH inhibitors suppress iodide concentration suggesting the importance of SH groups in the trapping of iodide by the thyroid.

Materials and Methods. Embryos used in this study were

obtained from fertile eggs from the experimental farms of the Texas A. and M. College. These eggs were collected at three-hour intervals in order to obtain as uniform an incubation period as possible. Although initiation of incubation cannot be said to be identical, allowing for variations in transit temperatures, nevertheless discrepancies have been relatively small. Since it was necessary to obtain several sections for the procedures to be followed, the glands from four to eight embryos at each age level were fixed and blocked together. The initial specimen was obtained the seventh day and subsequent specimens progressively at ¼ day intervals to nine days, then daily through 21 days. For the sulfhydryl studies, tissues were fixed in acid alcohol to prevent air oxidation of SH groups (Ethanol 80 percent, Trichloracetic 1 percent). These were embedded in paraffin following the routine histological procedure, sectioned at 7–8 mu, passed through xylene and graded alcohols to water and treated with DDD and Diazo Blue B according to the procedure of Barnnett and Seligman. (1) A set of four sections was obtained on each tissue, *i.e.*, sulfhydryl, disulfide, SS-SH and a blank. These were read as positive or negative—no attempt at quantitation being made beyond noting that a deep purple color (dicoupling) indicated an excessive amount of reactive material whereas a red color (monocoupling) suggested somewhat fewer available groups. A second series of embryo sections was obtained in the same manner and submitted to the periodic acid-Schiff reaction, following the procedure of McManus (1948), to be used as a guide to point up morphological changes, since this gives a very good picture of the first colloid droplets. These appear between 8¾ and 9 days to assume the characteristic colloid stain of this method. Studies have also been made of some surgical tissues from the thyroid to compare the chemical groups with the pattern presented in embryonic development.

Results. Resulting sections demonstrated the presence of combined SS-SH groups detectable in the thyroid colloid at 8¾ days, though rare. None were found below this. This situation remained until the 11th day when disulfide groups were first found. At some point in the following 24 hours sulfhydryl groups appear. In the 9 day section subjected to PAS, colloid droplets are readily detectable, though there is some variation

in the sections on the same slide, *i.e.*, some glands show few or none. However, it has been previously pointed out that zero incubation hour cannot be definitely timed and small time variations at this level may span the critical period. At this point the characteristic thyroid architecture is not in evidence, although some 24-36 hours later there is beginning indication of the cells tending to form the first small follicles. After the 13th day the pattern of development is set and by the 18th day it is somewhat difficult and often impossible to differentiate the subsequent embryo picture from that of the post-hatched specimens. From the series of surgical specimens from human thyroids there are 3-4 which show sulfhydryl reactions reminiscent of the 10th day of embryonic development. These show fairly good SS-SH reactions and doubtful or negative sulfhydryl and disulfide stains.

Closing remarks. This study was suggested in a sense by the work of Wegelin (1926) who pointed out that thyroid tumors might be more correctly classified pathologically by reference to their similarity to the various pictures presented in embryonic thyroid development. Thus, "struma trabecularis" would parallel the histologic structure seen up to the 10th day with the embryonic thyroid cells lying in sheets, "struma tubularis" when the cells have arranged themselves into the long tubes present before the final pinching off occurs to give the mature thyroid follicles (struma follicularis). It has been shown here (1954) that development of cells in tissue culture may be reversible. That is, having acquired progressively the ability to manufacture monoiodotyrosine, diiodotyrosine and thyroxine, the gland may lose the power to produce the latter two of these.

Fugo (1940), working with hypophysectomized embryos, found morphologic evidence of "regression" setting in at about the 14th day. If we postulate the presence of enzymes essential for each step of the process necessary for the manufacture of thyroxine, can we hope to presume that by identifying these enzymes and the dates of their appearances, we may at some time be able to supply substances which might be capable of stimulating immature cells through their normal phases of development to the ultimate goal of activity as mature units for function and production of the thyroid hormone.

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ENZYMATIC PROPERTIES OF TUMORS

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The biochemical literature contains a wealth of data on the enzymatic features of neoplastic tissues. However, the differences between normal and malignant tissues are not exactly striking, except perhaps in the case of enzymes involved in aerobic and anaerobic glycolysis. In this presentation I shall deal only with those enzymatic properties of tumors which can be studied histochemically.

I doubt very much whether the diagnosis of neoplasia can ever be made in a tissue sample by biochemical analysis only, in spite of the differences referred to. The same applies to histochemical studies. With the present methods, neoplastic changes cannot be recognized by histochemical means only. In general, it may be said that tumor tissue often possesses some enzymatic property which is rather characteristic of neoplasia as such. In other respects, however, most tumors, at least the better differentiated ones, simply imitate the enzymatic behavior of the parent tissue. The latter fact may contribute to the identification of the origin of a tumor when this is impossible on the basis of morphological criteria alone.

Let us take one by one the enzymes which have been investigated histochemically. Instead of giving you a comprehensive review of the subject, I shall report on my own material, largely unpublished. I shall refer to the literature only when I lack personal experience as in the case of succinic dehydrogenase.

1) Succinic dehydrogenase. According to Black, Kleiner, and Speer, malignant cells tend to retain the high activity of the parent tissue, and the staining may help to visualize invasion by tumor cells. Mackenzie and Fuller noted large globular areas of activity in some cells of vaginal smears from cases of carcinoma, but they refrained from identifying these cells with the ma[l]ig-

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nant ones. Leukemic blood cells do not distinguish themselves by abnormal activity (Wachstein).

2) Alkaline phosphates. The most active normal tissues are bone and intestinal mucosa. Among neoplasms, the most constantly and intensely active ones are osteogenic sarcomas, and this may be a diagnostic feature. There is a type of osteogenic tumor, formed by small, rather uniform, round or oval cells (Ewing's sarcoma; some synovioma-like tumors). It may be very hard to recognize this group, especially in small biopsy specimens, and they are often mistaken for neuroblastoma. However, the latter is invariably negative for alkaline phosphatase. Two cases will be shown in which diagnosis could be established by enzymatic methods.

Case 1). A 5 year old girl had a tumor mass, invading and destroying the head of the left fibula. Biopsy showed a monomorphous tumor, composed of round or slightly oval cells. At the very periphery of the specimen the cells tended to line tiny spaces. The alkaline phosphatase reaction was intensely positive. The leg was amputated but the patient died a short time later from metastases. The final diagnosis was Ewing's tumor or round cell sarcoma with a tendency to differentiate in the direction of synovioma.

Case 2). A 13 year old girl had a destructive lesion in the right humerus. Morphologically, the biopsy specimen looked much like the one in the first case; however, the tumor cells were entirely devoid of alkaline phosphatase. The diagnosis of non-osteogenic tumor, probably neuroblastoma, was made. The patient died shortly after, and at autopsy a neuroblastoma arising from the sympathetic chain was found.

Besides osteogenic tumors, intense phosphatase activity may be found in adenocarcinomas of the small bowel and in adrenal cortical tumors.

3). Acid phosphatase. Moderate amounts of this enzyme are present in practically all tissues, but the adult prostate and most prostatic carcinomas are tremendously active. This feature could be an excellent diagnostic aid if the histochemical method were dependable enough, which it is not. However, in the case of success, a distinctly positive reaction after 1½ to 2 hours of incubation is practically diagnostic of prostatic origin. Two cases

will be shown in which the diagnosis of prostatic cancer, metastatic to the skin, and to lymph nodes, respectively, could be made histochemically. Glück reported a few years ago that most cancers of the breast are also significantly active. This is an interesting observation, and, if proven to be true, it may render the determination of the nature and origin of breast tumors arising in men treated with estrogen for cancer of the prostate impossible by histochemical means.

4). Phosphamidase. This is also a widely distributed enzyme but the normal brain and over 90 per cent of all malignant epithelial tumors have an exceptionally high activity. Unfortunately, the histochemical method is somewhat tricky, and failures are common. However, a successful slide is a delight to the pathologist's eye. Malignant tumors are outlined sharply against the pale background of normal tissue, and every single invading malignant cell can be recognized as such by its intense reaction. Practically all epithelial malignancies react, with the exception of most renal carcinomas which are negative. Among sarcomas, some react but the majority are inactive. In addition to distinctly malignant processes, two essentially benign conditions are also intensely reactive: polyps of the colon and the so-called pale cell cysts of the breast which are related to apocrine sweat glands.

While the phosphamidase technique may be valuable in the detection of invasion by malignant cells, its use in diagnosis is of limited value. The intensity of the reaction usually parallels the morphological criteria of malignancy and adds little to the latter. In my experiments, the method does not work in smears, although Ebner and Strecker find it useful.

5). Esterase. This is not one enzyme but a large group of more or less closely related enzymes with somewhat different individual substrate specificities and sensitivities to activators and inhibitors. I cannot go here into the complicated problem of their classification.

There are several types of histochemically useful substrates. The Tween method is outmoded and too little sensitive; some other methods require expensive and hard to obtain substrates. For general purposes, naphtholic esters are recommended. Probably the most useful general substrate is naphthol AS acetate.

This is hydrolyzed by a large number of tissues, and the end result is a brilliant carmine-red staining.

As mentioned, esterases are present in many tissues but the largest amounts are found in the liver, the pancreatic acini, the chief cells of the gastric mucosa; somewhat lower activity is exhibited by the convoluted tubules of the kidney, bronchial mucosa, thyroid, apocrine sweat glands, and other tissues. Under embryonic conditions, the epithelium of the esophageal mucosa is highly active; in the adult, much less so, if at all.

The enzymatic behavior of tumors sometimes duplicates that of the parent tissue, sometimes not. Hepatomas yield intensely positive reactions; cholangiomas and metastatic carcinomas of the liver usually yield negative results. Gastric carcinomas, surprisingly, are invariably either negative or weakly positive. Squamous carcinomas of the esophagus and the bronchi are often intensely positive. When a bronchogenic carcinoma invades the pleura, the large esterase-positive cancer cells in the pleural tap present a spectacular and diagnostic picture. Similar carcinomas arising from the oral mucosa are sometimes fairly active. On the other hand, squamous cancers of the skin are invariably negative. Tumors of the breast behave erratically. Most of them are completely negative; rare instances may show an extensive general reaction. Numerous cases are seen in which highly active cells are scattered among a majority of morphologically identical but inactive cells. Tumors of the thyroid often retain the typical enzymatic distribution pattern of the normal thyroid in that esterase is localized in the basement membrane and/or in the basal portion of the cell. Renal carcinomas are negative or moderately positive.

By the use of inhibitors and activators, at least three different types of esterase can be distinguished in human organs. They are as follows: a) hepatic type; inhibited by fluoride, di-isopropyl-fluorophosphate (DFP) and anionic detergents; b) pancreatic type; not inhibited by fluoride but inhibited by DFP and actually activated by anionic detergents; and c) a general type, present in most other tissues, insensitive to fluoride and DFP but inhibited by anionic detergents. These differences will be shown in gross slides, containing a piece each of liver, pancreas, stomach, kidney and pituitary.

Case 3). A 5 year old girl had a large tumor mass involving the entire root of the mesentery, the back surface of the liver and the proximal half of the pancreas. Microscopically, the tumor consisted of fairly large, polyhedral, rather clear cells which contained no glycogen. It was impossible to decide whether it originated in the liver or pancreas; no other tumor was found which could be considered primary.

Histochemically, the tumor showed a very intense esterase reaction. When inhibitors were used, it was found that the esterase of the tumor was entirely insensitive to fluoride, totally inhibited by DFP and somewhat activated by anionic detergents. These features clearly classified it as pancreatic esterase. On the basis of these findings the tentative diagnosis of embryonic carcinoma of the pancreas was made.

One more hydrolytic enzyme is mentioned in the biochemical literature as fairly specific of tumors, beta-glucuronidase. Histochemical techniques published for this enzyme are grossly unsatisfactory, at least for human tissues. I have no knowledge of any histochemical studies on the presence of this enzyme in tumors.

Several histochemists are actively engaged in attempts at devising techniques for an increasing number of enzymes. If we are lucky, one day a method will be found the results of which will give us real help in the diagnosis and treatment of neoplasia.

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THE SIGNIFICANCE OF HISTOCHEMICAL TECHNIQUE IN CANCER RESEARCH

G. GOMORI*

In practically all fields of biological research which have both a morphological and chemical aspect, histochemistry is beginning to play an increasingly important role. The rising popularity of histochemistry is evidenced by the fact that in 1951 there existed only one, rather antiquated, textbook of histochemistry while today there are not fewer than five new ones available, with a sixth one in preparation. In addition, we have now three journals devoted to the subject, all born within the past three years.

The great interest in this young science is explained by its strategic position of straddling micromorphology and chemistry (biochemistry). Of course, data about the chemical composition of various tissues have been available for over a century; however, the identification and localization of a large number of chemical substances, especially enzymes, on a microscopic scale, has become possible only lately.

In this introduction to "Histochemistry in Cancer Research" I shall try to say a few words about some important technical aspects of histochemical research in general. The papers to be presented in the course of the meeting will deal with practical applications of histochemistry to various specific problems.

The techniques used in histochemistry can be divided in two large classes, the statistical ones and those of direct microscopic visualization. The statistical ones, of which the most typical examples are the methods developed by Linderstrøm-Lang and his group, used different but comparable tissue samples for microscopic examination and for chemical analysis, respectively. They are actually a form of quantitative microanalysis by test tube methods. On the other hand, methods of direct visualization use only one tissue sample and observe under the microscope chemical reactions which occur *in situ*. These two types of methods

* From the Department of Medicine, The University of Chicago. Presented at the Annual Symposium on Cancer Research held at the M. D. Anderson Hospital and Tumor Institute, Houston, Texas, March 15, 1955.

both have their specific advantages and shortcomings but can supplement each other in a most convenient way. In the statistical methods, quantitation is excellent but localization is often insecure or actually impossible unless the chemical constituent looked for is localized in only one type of morphological element, readily identifiable and quantitatable under the microscope. This condition is not too often fulfilled because specific substances are often localized in more than one type of morphological structure or may be present in structures which do not lend themselves readily to quantitation (connective tissue, vessel walls, blood, etc.). With microbiochemical methods, good results may be obtained when the substance investigated occurs in a widespread way but at a local concentration too low for the sensitivity of the direct method. On the other hand, quantitation by direct visualizing methods is poor; in fact, often misleading, especially to the more naive investigator. The optimal field of application of the direct methods is those instances where the substance is present in small, discontinuous, widely scattered areas of high local concentration. In such cases the dilution factor may render test tube readings so low as to be worthless.

There can be no doubt that for quantitation test tube methods are preferable. However, adherents of these methods have often made the claim that with the direct methods even localization is on a shaky basis since one can never be sure that precipitation will actually occur strictly *in situ* and that the result seen is not due to secondary absorption of reaction products. This may be the case with some techniques where the reaction rate is too slow or precipitation is initiated under non-optimal conditions. In most accepted methods, however, there is excellent evidence available that localization is accurate within the resolving power of high dry objectives. Therefore, as far as localization is concerned, the direct methods are always preferable whenever feasible. Unfortunately, for some substances it has been impossible so far to devise suitable *in situ* reactions. Other substances, especially some of the more delicate enzymes, will not tolerate the manipulations necessary to obtain sections. However, certain refinements of the *in situ* technique such as freezing-drying or sectioning in the cryostat may expand the applicability of the *in situ* methods to some extent.

A kind of combination of methods of direct visualization with those of quantitation by extracting the dyestuff deposited in the sections, proposed and practiced by Doyle of Chicago, appears to offer the best compromise solution of the problem, whenever practicable.

Here I wish to issue a note of warning against expecting too much from two types of technique which, in my opinion, have enjoyed excessive and undeserved confidence. The first type of technique is freezing-drying and its modifications. It is usually argued that they are extremely gentle, especially to enzymes, since they avoid the most harmful step, fixation. This is true but it is not the whole truth. Frozen-dried tissues, being unfixed, contain their proteins and other colloidal substances in a seminative, freely soluble form. They may be lost from the tissue promptly when the sections are transferred into an aqueous medium. Concentrated salt solutions, used to keep proteins precipitated, often are strong inhibitors of enzymes and, in addition, poor solvents for many of the substrates. To avoid the losses mentioned and to prevent various diffusion artifacts, it is necessary to fix the sections and to denature the soluble proteins. This, of course, would appear at first glance to demolish all the advantages of the freezing-drying method. However, the case is not one of an all-or-none effect. The thin sections obtained by freezing-drying have to be fixed for only about a minute while regular tissue blocks have to go through a long passage of dehydrating agents, and the procedure may take days. It may not be irrelevant to an alcohol-sensitive enzyme whether it is exposed to alcohol for one minute or for three days. One more thing should be mentioned: some enzymes may be actually more sensitive to drying out than to certain forms of fixation. For instance, cholinesterases of the rat are far more active in formalin-fixed frozen sections than in frozen-dried ones.

My second warning concerns the interpretation of results obtained with the use of enzymes. Up until recently, the removal of a substance from a section by specific enzymatic action was considered a *prima facie* evidence for the identity of the substance removed, with the substrate of the enzyme employed. This is permissible only if the enzyme used is of good specificity and purity. Abolition of basophilia by very dilute solutions of ribo-

nuclease or desoxyribonuclease can still be considered reliable evidence for the corresponding nucleic acids being responsible for the initial basophilia. However, the situation is entirely different with enzymes which hydrolyze various carbohydrates. I have in mind specifically the case of hyaluronidase, pectinase and the like. These enzymes are extremely crude, and their specificity is undetermined. Benditt and French have shown that they may cause their results by at least three different mechanisms of which only one is specific.

In summary I may say that there are a number of sources of error in histochemical techniques. However, in due time, careful critical analysis of methods proposed will, no doubt, establish the soundness of some and the unreliability of others, and will help to perfect partially good techniques. There is every reason to believe that in a few years histochemistry will possess a large stock of thoroughly dependable, well standardized techniques which should contribute in a spectacular fashion to our present knowledge of the chemistry of the tissues under various conditions.

THE EFFECT OF METHYLCHOLANTHRENE ON THE DEVELOPMENT OF THE CHICK FROM THE EMBRYONIC STAGE*

P. O'B. MONTGOMERY

Methylcholanthrene is a powerful carcinogenic agent in many adult animals. Its effect on developing embryos and animals have not been studied. The purpose of this paper is to report the effects of methylcholanthrene on developing chick embryos and upon the chicks which were hatched from these embryos.

Methods. Fertile White Rock eggs obtained on the 8th, 12th and 16th days of incubation were used.[1] Each egg was opened and 1 mgm. of methylcholanthrene was scattered at random on the chorioallantoic membrane. The defect in the egg was covered with scotch tape and the eggs returned to the incubator where they were kept under constant conditions of temperature, humidity and rotation until hatching. The day after hatching the chicks were weighed and transferred to a chicken brooder where they were fed chick starter and water *ad libitum*. They were weighed at the same time each day thereafter for fourteen consecutive days. Eighty-two normal chicks were obtained the day after hatching and were weighed and kept under identical circumstances to serve as a control group.

Results

The results are given in tables 1, 2, and 3. Two features appear worthy of comment. First, the majority of the chicks in each group failed to hatch or did not live one day. The eggs which did not hatch were opened and grossly normal 21 day chicks were present in each case. These chicks apparently lacked the strength to hatch. In group I, 42 chicks hatched out of 182 eggs, but 36 of these failed to live 12 hours. In group II, 98 chicks

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[1] The fertile eggs were obtained from the Western Hatcheries, Dallas, Texas.

hatched out of 200 eggs, but only 23 survived 24 hours and only 8 survived more than 48 hours. Of the chicks in groups I and II which hatched and died, nearly all showed a generalized weakness, as well as a deformity of the position of the legs and feet. The legs were held in rigid extension with the toes in a position of flexion. These chicks were usually not able to stand; or, if able to stand, not able to uncurl their toes. This group of chicks was

TABLE I

1 MGM. METHYLCHOLANTHRENE - 8 DAY EGGS			82 NORMAL CHICKS AVERAGE DAILY WEIGHT 1 - 14 DAYS
NUMBER OF EGGS	NUMBER OF CHICKS	AVERAGE DAILY WEIGHT 1 - 14 DAYS	
192	7	36.7	47.7
		34.4	44.4
		41.3	54.7
		41.8	56.3
		51.7	63.6
		51.5	68.5
		59.0	69.4
		62.7	75.3
		61.6	76.6
		78.7	85.2
		83.3	96.6
		84.6	97.2
		103.5	151.2
		105.8	164.4

also characterized by the presence of a covering of a gelatinous, gummy, light green membrane which had to be manually removed in order for hatching to be completed. Of the chicks in group III which hatched and died, only a very few showed any of the preceding changes.

Second, the average weight gain in groups I and II as compared to the normal is considerably reduced over the fourteen day test period. This is not true in group III, where the average weight gain is comparable to the normal. Figures I, II, and III depict a normal chick on the right and a methylcholanthrene-treated chick on the left at 2, 8 and 10 days. These photographs illustrate the striking lack of growth in the methylcholanthrene-treated chick compared to the normal.

TABLE II

1 MGM. METHYLCHOLANTHRENE - 12 DAY EGGS

NUMBER OF EGGS	NUMBER OF CHICKS	AVERAGE DAILY WEIGHT 1 - 14 DAYS	82 NORMAL CHICKS AVERAGE DAILY WEIGHT 1 - 14 DAYS
200	23	35.3	47.7
	7	36.4	44.4
		37.5	54.3
		38.6	56.3
		39.7	63.6
		45.0	68.5
		47.8	69.4
		49.5	75.3
		53.1	76.6
		62.0	85.2
		69.7	96.6
		72.2	97.2
		75.9	151.2
	6	88.0	164.4

TABLE III

1 MGM. METHYLCHOLANTHRENE - 16 DAY EGGS

NUMBER OF EGGS	NUMBER OF CHICKS	AVERAGE DAILY WEIGHT 1 - 14 DAYS	82 NORMAL CHICKS AVERAGE DAILY WEIGHT 1 - 14 DAYS
180	78	46.6	47.7
		49.6	44.4
		54.4	54.7
		59.6	56.3
		58.4	63.6
		73.7	68.5
		66.8	69.4
		73.3	75.3
		89.7	76.6
		107.8	85.2
		116.8	96.6
		127.7	97.2
		129.9	151.2
		147.5	164.4

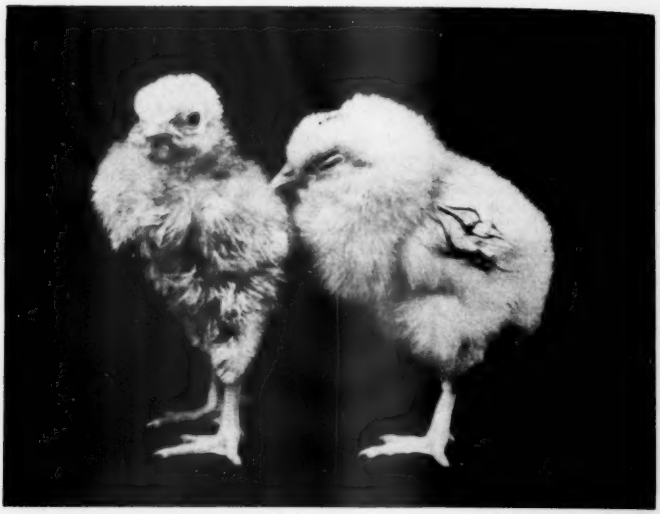

FIG. I. Two-day-old chicks. Chick on left—methylcholanthrene applied on 12th day of incubation. Normal chick on right.

FIG. II. Eight-day-old chicks. Chick on left—methylcholanthrene applied on 12th day of inoculation. Normal chick on right.

Discussion. No explanation is apparent for the observed effects of methylcholanthrene when applied in this fashion to the developing chick embryo. Lack of dietary intake does not seem to be a factor. The treated chicks were observed to eat plentifully and practically always had full craws during the daylight hours. Post mortem examinations on the chicks which failed to hatch and on the chicks which died did not reveal any significant gross changes.

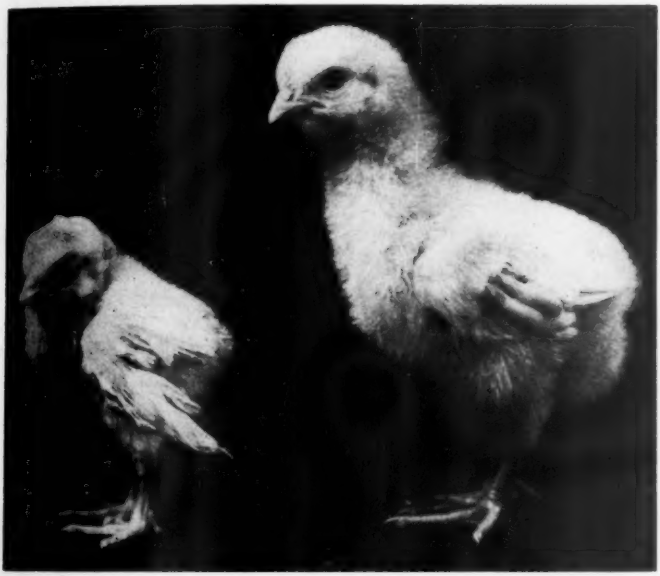

FIG. III. Ten-day-old chicks. Treated 12-day embryo on left. Normal chick on right.

A general toxic effect does not appear to be a sole factor. Haddow *et al.* (1937, 1939) report that many carcinogenic compounds are general growth inhibitors when tested in young rats. They did not find this property shared by non-carcinogenic systemic poisons.

It is interesting to note that the chicks in group III which lived did not show retardation of growth. Two possible explanations of this may be considered. Either the methylcholanthrene was

not absorbed prior to hatching in group III; or the inhibitory effect only occurs if the methylcholanthrene is applied before the 16th day. The former explanation does not appear to be too satisfactory in view of the 50 percent mortality at hatching time in group III. Further evidence that carcinogens are metabolized by embryos may be found in the report of Chalmers and Peacock (1936). These authors report that other carcinogens injected into 18-day chick embryos disappear within a few days. The few chicks allowed to hatch in this study were reported to be normal.

Summary. The effect of methylcholanthrene on the development of chick embryos and chicks is presented.

If applied before the 16th day the chief effects are failure of hatching in the majority of instances and in the case of hatching, retardation of growth.

Acknowledgment

I wish to acknowledge the technical assistance of Mrs. Ovia Walker, and to thank Mrs. Walker and Mr. Sage for the photographs.

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SOME METABOLIC ASPECTS OF MULTIPLICATIVE GROWTH*

WIKTOR W. NOWINSKI

To Professor Dr. Fritz Baltzer, with admiration and in friendship on the occasion of his 70th birthday.
W. W. N.

In studies on the relations between enzymatic activity and growth, in a growing organ it is extremely difficult to discriminate between enzymatic patterns connected with the maintenance of the living processes and those which are essential for the growth process. To overcome this difficulty it seemed to us that the developing wing and leg buds of the chick embryo present ideal materials. In certain early stages of ontogenesis (days 3 and 4) both appendages are of the same size (Hamburger and Hamilton, 1951) and grow at the same rate; however from approximately the fifth day of development onward, the rate of growth changes: the wings grow slower and the legs—faster. Our premise, therefore, was that the rate of activity of enzymes concerned with the phenomenon of growth, when measured at different stages, should closely follow the rates of growth of the two appendages, *i.e.*, it should measure less in the wings and more in the legs.

Another point which is often neglected and confuses the results of such studies is the fact that the growth is a complex phenomenon, consisting of three different components (Needham, 1942): (1) growth by increase of cell number (multiplicative); (2) growth by increase in cell size (auxetic), and (3) growth by increase of the intercellular space (accritionary). Although the first component is the prevalent one, there is little doubt that the other two also need enzymatic activity for their realization. Therefore in studying metabolic patterns concerned with growth, it is of paramount importance to investigate these three types separately. This requirement is not always considered.

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We decided to study first the enzymes connected with multiplicative growth. The material used in the work was suggested by Doctor John Russell Couch of the Texas A. and M. College. It consisted of a cross: White Leghorn ♀ × New Hampshire Red ♂. This very vigorous hybrid proved to be an ideal material for the study of growth.[1]

In order to establish the increase in cell number at consecutive stages of development of the wings and legs, we chose the accumulation of DNA as a criterion. In order to establish this, it was necessary first to determine the mean average content of DNA per nucleus ("constancy") in the wings and legs during ontogenesis. The figures, obtained with Dounce's method of isolation of the nuclei (Dounce, 1943) and Schneider's method (Schneider, 1945) of extraction of the DNA (which was determined by the diphenylamine reagent), showed that there is no significant difference in the DNA content per million nuclei at different stages and that the mean average amounts to 8.2 μg in both appendages. The mean standard deviations were ± 1.2 for the legs and ± 1.4 for the wings (Nowinski and Yushok, 1953). Cytological studies, carried out by Doctor T. C. Hsu,[2] showed that the mitoses are not concentrated on the tip, but that they are distributed along the whole growing appendage.

Based on these facts, the DNA accumulation at stages from the fifth to twelfth day were determined (Nowinski and Yushok, 1951, 1953). The figures thus obtained showed very similar values on the sixth day (11.8 μg DNA per wings and 13.3 μg per leg); however, in the course of further development, the accumulation of DNA showed progressive differences, reading on the twelfth day 389 μg DNA per wing and 863 μg DNA for the leg (Table 1).

When the accumulation of DNA is plotted against different stages of development (in days), an exponential curve is obtained. When this curve is compared with a curve of the overall growth as expressed by the wet weight, a near parallelism is seen between these two curves up to the tenth day; this means

[1] We once more thank Dr. J. R. Couch for the suggestion and Dr. J. Quisenberry, Head of the Poultry Husbandry Division of Texas A. and M. College, for supplying us with this material bred under his personal supervision.

[2] We express our sincere thanks to Dr. T. C. Hsu, who kindly investigated our material cytologically.

that up until this stage, growth takes place primarily by an increase in the number of cells. From the tenth day on, the curves of the overall growth and of DNA accumulation approach each other. This indicates that at these stages, the auxetic and accretionary types of growth participate more in the growth process than during earlier stages. On semi-logarithmic paper, the data present a nearly straight line.

TABLE 1

Accumulation of Desoxyribosenucleic Acid in the Developing Wings and Legs
(From Nowinski and Yushok, 1953, modified)

Days of Incubation	μg DNA per Appendage (with mean standard error of pooled samples)	
	Wings	Legs
5	11.8 ± 0.66	13.3 ± 0.60
6	18.6 ± 0.76	24.8 ± 0.70
7	25.5 ± 1.1	48.1 ± 1.5
8	48.1 ± 1.4	92.6 ± 4.0
9	81.5 ± 2.7	152 ± 3.2
10	127 ± 1.5	271 ± 7.0
11	230 ± 8.7	508 ± 8.4
12	389 ± 11	863 ± 35

Calculation of the instantaneous rate of accumulation of DNA was carried out by use of Brody's equation (Brody, 1945):

$$\text{Kdna} = \frac{100\ (\log_e W_2 - \log_e W_1)}{t}$$

where W_2 and W_1 are the final and initial amounts of DNA and t is the interval of time (24 hours). The results of these calculations are summarized in table 2. This table shows that between the fifth and eighth day of incubation, the legs accumulate DNA at a much higher rate than the wings. This follows particularly clearly from the comparison of the ratio of rates, leg/wing. It drops then on the 8-9 day period, increases again, and from the 9th till the 12th day it is practically equal. We decided to restrict our metabolic studies to the period between the 6th and 8th day only, adding the 8-9 day interval as a control: at this stage the ratio of rates drops to 0.94 as compared to 1.3 of the preceding day; it was assumed that the enzymatic activity concerned with growth accordingly will be lower at this stage.

As a reference for our metabolic studies we have chosen 100 μg DNA, which—in our material—corresponds to approximately twelve million cells. This unit actually represents a population of cells in which the different types of tissues are evenly distributed. By comparing the data of a fast-growing population (legs) with a slower-growing one (wings), we attempted to evaluate the prevalent metabolic patterns characteristic of the several changes in the growth rates of these two appendages. In other words, our approach is comparable to the approach of a statistician in comparing the growth of two populations. From such a comparison he evaluates the causes, conditions and rules which control the demographic differences of the populations.

TABLE 2

Instantaneous Rate of Accumulation (Kdna) of DNA and Ratio of Rates
(After Nowinski and Yushok, 1953, modified)

Incubation Period Day	Rate of DNA Accumulation in Percent Per Day		Ratio of Rates
	Wings	Legs	Leg/Wing
5–6	46	62	1.4
6–7	43	66	1.5
7–8	52	66	1.3
8–9	53	50	0.94
9–10	44	58	1.3
10–11	59	63	1.1
11–12	53	53	1.0

We chose two types of metabolism for study: oxidations and anaerobic glycolysis.

In the study of oxidations, as measured by the oxygen uptake with the Warburg technique and using glucose for substrate, we found that the amount of oxygen consumed per appendage increases in the wing from 1.85 μl per hour on the fifth day to 47.4 on the eleventh day, whereas in the leg the increase is 2.35 μl to 93.6 μl for the same stages (Yushok and Nowinski, 1952, also unpublished data). These results were predictable because the uptake of oxygen must parallel the increase in bulk of the appendage. If these results are calculated on the basis of dry weight, there is a steady decrease of oxygen consumption from Q_{O_2}—12.0 for wings and —11.6 for legs on the fifth day to —8.07 (wings) and 7.39 (legs) on the tenth day. Statistical evaluation of the results shows that the differences for the corresponding days

are not significant. This observation accords with the known fact that, with the progress of ontogenesis, oxidations decrease when calculated on either dry or wet weight basis (Needham, 1931; Romanoff, 1943). However, if the data are calculated on the cell population basis, *ie*.. on 100μg DNA, the amount of oxygen taken up is constant throughout the investigated stages, again without any significant difference in the oxygen uptake of the wings compared to that of the legs. From these experiments the conclusion must be drawn that cells at a higher rate of division do not use more oxygen than the slower-dividing ones, *i.e*., that the oxygen consumption is not connected with the multiplicate growth.

Our work on anaerobic glycolysis, as measured by the Warburg technique, shows that, on the appendage basis, there is a steady increase in CO_2 formation similar to the case of oxidations. The range is from 2.76 μl for the wings and 3.20 μl for the legs on the fifth day to 30.1 μl (wings) and 59.1 μl (legs) on the tenth day. Calculated on the dry weight basis, the $Q^{N_2}_{CO_2}$ shows a steady decrease, a fact already known for total embryos (Warburg, Posener and Negelein, 1924; Needham and Nowinski, 1937; Nowinski, 1939). When the decrease in the CO_2 formation of the wings is compared with that of the legs, no significant difference is present. However, when the data are calculated on the cell population basis (100 μg DNA) there is a significant drop of CO_2 production in the wings, from 30.6 μl to 21.1 μl, between the fifth and sixth day. Then it becomes stationary until the ninth day, and finally drops significantly to 20.6 μl on the tenth day. A similar tendency is manifested in the anaerobic glycolysis of the legs: it drops from 30.4 μl on the fifth day to 20.0 μl on the tenth day, with a stationary period between the sixth and ninth days.

If significance is estimated between the corresponding stages of the wings and legs, a difference is obtained on the sixth and seventh days ($t = 4.1$ and 4.7 respectively). These stages correspond to the highest ratio of rates (Table 1).

In figure I are summarized our results on oxygen uptake and anaerobic glycolysis at the investigated stages, together with a graphic representation of the ratio of rates. From this figure the

relations between these metabolic processes and the rate of development of wings and legs can be easily followed.

The fact that there exists a parallelism between the high rate of growth and anaerobic glycolysis justifies the conclusion that glycolysis is associated with multiplicative growth rate. We also found (unpublished data) that anaerobically glu-

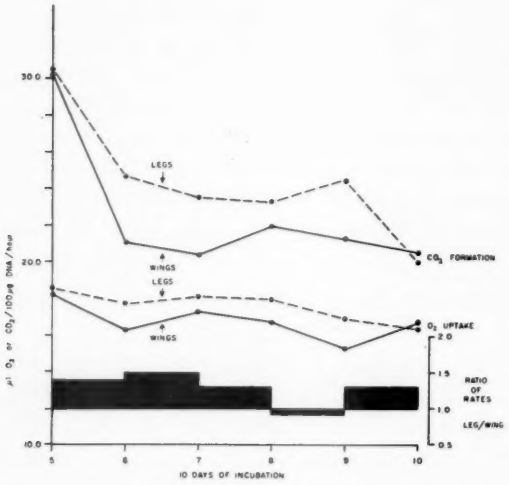

FIG. 1. Relations between respiration, anaerobic glycolysis and rate of rates in developing limb buds of the chick embryo, calculated on the basis of 100 μg DNA per hour.

cose uptake is much lower than aerobic uptake and that the production of lactic acid is smaller in the presence of oxygen. This suggests that, aerobically, most of the glucose used is going into the Krebs cycle. It was found, in fact, that the addition of a series of intermediates of this cycle increases the oxygen uptake (Nowinski and Partridge). Calculated on a Q_{O_2}—basis no significant difference has been found between the appendages. However when calculated on the 100 μg DNA-basis the difference immediately became manifest. So, for instance, succinate on the sixth day of incubation shows an increase from 14.20 μl/ 100 μg DNA/ hour to 16.58 μl/ 100 μg DNA/ hour: the significance of the difference of means is 3.8.

The differences, obtained with anaerobic glycolysis seem to us to be due to the energy requirement connected with synthesis of high energy phosphorous bonds. This energy is necessary for a series of processes in the growing cell, of which the synthesis of proteins is one of the most important. Furthermore, Lettré's experiments (Lettré, 1951, 1952) strongly suggest that the contraction of the spindle in anaphase and that possibly the movements of the cell protoplasm draw their energy also from this source. Of course, from the point of view of energy production, the anaerobic glycolysis, where only two molecules of adenosine triphosphate (ATP) are formed, is not very economical. In this respect, areobic glycolysis, where pyruvic acid, instead of being converted into lactic acid (as in anaerobiosis), to a large extent enters the Krebs cycle, is much more desirable. Within this cycle forty molecules of ATP are formed. We hope that work being carried out in our laboratory at present consisting of a study of Krebs cycle and of transaminases connected with it will help illuminate our basic problem.

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DO NUCLEI OF LIVING CELLS CONTAIN MORE DNA THAN IS REVEALED BY THE FEULGEN STAIN

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A few years ago a famous English cytologist said that he thought the time had come when cytologists should boldly advance hypotheses to explain, or to correlate, information obtained from different fields of research, with a full realization that subsequently, in the light of additional or new information, it would probably be necessary either to discard the explanation or to greatly modify it! In the meantime, however, research workers would be stimulated to verify or to disprove the hypothesis and more rapid progress in cytology would thus be assured. It is somewhat in this spirit that I appear on the program this afternoon for it appears to me that there is a considerable body of knowledge about various aspects of nucleic acid, some cytological, some chemical, which needs to be integrated or, at least, we need to consider the several possibilities which should be explored in order to correlate conflicting data and concepts. When one goes far afield from his own specialty there is a danger, of course, that inadvertently he will not know of and fail to mention papers pertinent to his discussion or that he will express ideas which others have advanced before without giving due credit. I hope I will not err too much in these ways.

At the present time there exist in cytological and cytochemical literature two points of view, which appear difficult to reconcile, regarding chromatin or the DNA in the nuclei of cells. According to one point of view, the amount of DNA in diploid cells of an individual, or other members of the same species, is constant (see Swift, 1954, for a review of the literature) and that in differentiated or non-dividing cells the DNA is extremely stable chemically as is indicated by the relatively small uptake, for example, of P^{32} as compared to other cell components. The other and older concept is that chromosomes contain two types of DNA, or that DNA is associated with two types of regions along the

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chromosome. One is called euchromatin, or gene chromatin. One would expect great constancy in the DNA component of such regions since genes are regarded as nucleo-proteins. The second type of DNA is found in the so-called heterochromatic regions. Heterochromatic regions differ from euchromatic areas in a number of ways. Genetically heterochromatic regions carry very few specific genes; considerable quantities of heterochromatin may be removed from cells without greatly affecting the individual and the overall genetic effect of this type of DNA is regarded as a generalized (metabolic) one. Morphologically, heterochromatic regions show a number of visible peculiarities such as remaining condensed at times when the euchromatic regions are greatly extended, non-specific pairing especially well observed in salivary gland nuclei and other features. Chemically, it has been found that the amount of DNA attached to heterochromatic regions can be greatly altered, for example, by temperature changes resulting (in the low range) in what Darlington (1940) calls "nucleic acid starvation." In this and other ways heterochromatin appears to be a more or less labile type of DNA. Many recent investigators think that heterochromatic regions have to do with nucleic acid metabolism and hence the DNA component might be expected to vary in amounts in a nucleus if different physiological states involved nucleic acid metabolism.

The concept of DNA constancy rests primarily upon microphotometric determinations of the amounts of Feulgen stained material in nuclei and to a lesser extent on biochemical evidence. As most of you are aware, in order to apply the Feulgen test for DNA it is necessary to hydrolize fixed tissues or sections in 1 N HCl at 60° C. for a short time in order to remove the purine bases thus unmasking aldehyde groups which combine chemically with the leucoform of basic fuchsin. Let me say at once that it is now generally agreed that Feulgen's method is specific for DNA when it is properly carried out. Further, I am not disposed to question the validity of the evidence obtained by the photospectrometric techniques. In earlier studies, for example, that of Pollister in 1952, the spread of values for DNA in individual nuclei was in the order of $\pm$ 15 per cent. But later refinements introduced by Patau (1952) and by Ornstein (1952) and

others have reduced instrumental errors and at our laboratory, in Austin, we are obtaining rather consistently values which vary from eight to ten per cent. It seems likely that such differences in values are instrumental and not due to wide fluctuations in DNA. In short, from personal contact with this method I am convinced that with due care for instrumental sources of error, very little variation will be found in the amount of DNA in diploid nuclei of tissues taken from the same animal.

Opposed to the concept of DNA constancy, as revealed by the photometric techniques, is a very considerable body of cytological and chemical evidence which points to extensive changes in the amounts of DNA found in the heterochromatic regions of certain cells. For example, during the early development of oocytes in the ovary of either a frog or toad small granules of Feulgen-positive material (DNA) become detached from heterochromatic regions of the chromosomes, small nucleoli very rich in RNA develop in contact with the DNA granules and then both components move toward the inner surface of the nuclear wall. As the nucleolus nears the nuclear wall the DNA granule attached to it decreases in size and when the wall is reached the nucleolus is resorbed. Concurrently, there appear in the cytoplasm of the oocyte quantities of RNA which is densest just adjacent to the nuclear wall. This sequence in events, the detachment of heterochromatic granules, the development of nucleoli rich in RNA, the migration to the nuclear wall and the resorption of nucleolar material with the concurrent appearance of RNA immediately outside the nuclear wall, all point to a causal relationship. Just what is the nature of the chemical changes involved is not known. Caspersson (1947) has suggested that substances may be released from the heterochromatin-nucleolar complex which diffuse through the nuclear sap and induce the formation of RNA in the cytoplasm. I have assumed that the DNA granules conditioned the formation of RNA in the nucleolus either by a direct conversion, which is suggested by the decrease in the size of the DNA granule, or in some indirect way (Painter and Taylor 1942). To me it has seemed simplest to assume that the RNA formed in the nucleolus diffuses through the nuclear sap and passes in molecules of relatively low weight or polymer through the nuclear wall to supply the cytoplasm with the RNA needed for

protein and possibly other syntheses incident to storing up reserve food stuff in the egg.

The example of the toad's egg illustrates the sort of cytological evidence which underpins the general concept of heterochromatin lability and its relationship with nucleic acid changes or metabolism. If the DNA of heterochromatic areas is actually used up in such nucleic acid changes, that is, if all or part of the DNA molecule is used up in forming RNA, then cells conspicuously rich in RNA, as those of the pancreas, should show considerable variation in the DNA content of nuclei during the course of a secretory cycle. This, however, does not appear to be true in tissue stained by the Feulgen method.

There have been a few investigators who report as much as a 20-fold variation in the amount of DNA in secretory cells, for example, Leuchtenberger and Schrader (1952); but Swift (1954) using similar material has shown that the DNA values in different nuclei fall into a 2-4-8 polyploid series and hence that endomitosis had probably occurred in the gland in question. Thus this evidence of variation in DNA was not valid.

This past fall there appeared a paper by Taylor and McMaster (1954) in which they report on the P^{32} uptake in germinal cells of the anthers of lilies. A variety of techniques were used including autoradiography, Geiger counts, nucleases and Feulgen's stain. Two conclusions in this paper concern us here. The first is that it was found (after removing or accounting for other phosphorus sources) that the amount of P^{32} associated with DNA varied greatly depending on the method of extraction. If the P^{32} is removed from the chromosomes by nucleases, from 20 to 85 per cent more P^{32} is obtained, than from cells which were first subjected to the acid hydrolysis, a normal step in staining with Feulgen's reagent. From this they conclude that growing cells contain an acid-labile type of DNA which is not revealed in Feulgen preparations. Needless to say, this conclusion, if verifled by later work, would readily explain, or reconcile, the concepts of DNA constancy and heterochromatin lability. It would appear that the Feulgen technique reveals primarily gene DNA, which would be expected both to be constant and stable chemically while other types of DNA might be affected by acid hydrolysis. Some four different types of heterochromatin are now

recognized on morphological behavior or genetic grounds and one or more of these might be acid-labile. And some of you will doubtless recall that Bendich (1952) is finding a number of types of DNA in his analyses of nuclei. The conclusion reached by Taylor and McMaster, therefore, is entirely tenable and is in line with some other evidence.

After reading the paper by Taylor and McMaster I carried out two series of experiments designed to indicate the nature of the material removed by acid hydrolysis and a third series of tests are now under way. About 3 grams of mouse cancer tissue (several neoplasms were used) was finely divided and preserved in Carnoy's fluid (one of the fixatives used by Taylor and McMaster). After the usual procedures, this tissue was hydrolyzed for about 6 minutes in 1N HCl at 60° C. The hydrolysate was then tested for the presence of thymine and thymidine by the bioassay technique. Significant amounts of either compound were not found in two separate runs and assays. It appears therefore that the acid hydrolysis employed does not remove any DNA in the form of thymine or thymidine.

A study has been made also of grasshopper chromosomes in the following way. A number of slides containing sections of grasshopper testes were prepared. All were subsequently treated with ribonuclease for 2 hours at 37° C. to remove all traces of RNA. Part of the slides were subjected to the standard acid hydrolysis, the unhydrolized slides serving as controls. All slides were stained in Toluidine Blue buffered to a pH of 5.9 (to avoid staining basic proteins). Upon microscopic examination it was not possible to distinguish any significant difference in the amount of stain taken up by the DNA on hydrolized or control slides. Admittedly this is a crude test but if the acid hydrolysis had removed large quantities of DNA some difference in the intensity of the stain would probably have been noticeable.

Within the past few years some three groups of biochemists (Barnum, *et al.*, 1953; Stevens, *et al.*, 1953; and Daoust, *et al.*, 1954) have measured the P^{32} uptake in mammalian tissues with active mitoses. Their results, so far as this discussion is concerned, add up to this: The P^{32} uptake during a single mitotic cycle is so high—at least twice that expected—as to indicate that in chromosome reduplication the process does not involve simply

the synthesis of a new DNA molecule on the stable template of the old DNA molecule but rather that both molecules (resulting from the reduplication) are "new" so that apparently the template itself undergoes change or is metabolized. The experiments cited give no hint as to the type of reorganization other than the phosphorous moiety.

Let us now turn to some well known facts about P^{32} uptake, in general, in mammalian tissues. In the intestinal mucosa of the rat, where mitoses are very abundant, the P^{32} turnover is reported to be 15 per cent in 24 hours by Hevesy and Ottesen (1943). In the case of the thymus gland of young rats, Andreasen and Ottesen (1945) report an uptake of about 8 per cent in 3 hours which if continued would amount to some 60 per cent in 24 hours. Now it is well known that there is some mitotic activity in the thymus but this is less pronounced than in intestinal mucosa. It seems clear, therefore, that the P^{32} uptake reported must involve, to a large extent, reactions of phosphorus which are not related to DNA reduplication.

The last point I wish to refer to before trying to bring some semblance of order from the facts reviewed is the Watson and Crick model of the DNA molecule. These workers believe that the DNA molecule consists of a double helix of two complementary chains with the phosphates on the outside of the helix and the nitrogen bases on the inside.

This model suggests that the position of the phosphorus on the outside of the chain without cross-linkage might favor its rapid exchange or replacement without disturbing the sugar and base moieties, that is, without disrupting the whole DNA molecule. If this were true, then it is possible to explain or correlate some of the facts to which I have referred earlier in this paper.

In the thymus gland, I visualize the DNA molecules as possibly acting, in part at least, as conversion centers, that is, they take phosphorus in some form from the environment, convert it to another form and release the new compound to the environment without a disruption of the rest of the DNA molecule. I am sure that the biochemists can cite many instances where somewhat similar exchanges take place. Not being a chemist I have no idea as to the nature of the substances being produced by the thymus but from the facts presented I would expect P to be

involved. I hope some chemist may find this question a challenging one.

If the exposed position of the phosphorus along the surface of the DNA molecule, facilities phosphorus metabolism—using this word in a general sense—then the excessive amounts of P^{32} found upon chromosome reduplication need not invalidate the template concept of nucleoprotein reduplication. One might suppose that in cells normally there is a certain amount of P turnover not associated with DNA reduplication, and that the excess in P^{32} observed reflects metabolic activity as well as DNA synthesis.

In a somewhat similar way, the phosphorus compounds which Taylor and McMaster report removed from DNA by acid hydrolysis may not have been in the form of nucleotides or even precursors of DNA, but some other product being formed through the mediation of the DNA molecules.

Now let us look once again at the question of DNA constancy and DNA chemical stability in non-dividing cells. As I have said, in my earlier papers, in order to give meaning to the sequence of events, the simple assumption was made that heterochromatic DNA was converted into RNA—an assumption which my biochemical friends regarded as unlikely. It still appears to me a tenable working hypothesis which should be thoroughly explored before it is discarded. The course of events in the toad's egg, that is, the initial detachment of heterochromatic granules and subsequent to this the appearance of nucleoli and of RNA, the latter first in nucleoli and later in the cytoplasm all point to such a conversion. And there is some evidence (for example, see Taylor and McMaster) that when P^{32} is made available to an organism, it is first incorporated into the DNA, or predominantly so. and later appears in the RNA of cells.

There is, however, another explanation possible which will explain the DNA constancy and the DNA-RNA interrelation. It is entirely conceivable that the DNA molecule is necessary, in some way, for the formation of RNA—that it mediates the production of ribose nucleic acid without being used up in the process. Or, to put it another way, the DNA serves as a template of sorts around which RNA molecules are synthesized, or that DNA

forms one or more necessary precursors of RNA. Here is a fertile area both of speculation and research inquiry.

In conclusion, and in justification for this paper, I return to my initial paragraph. We must boldly build hypotheses and let others know about them and then assiduously follow up the leads suggested. In my laboratory we are busily engaged in investigations suggested by my speculations, and I hope that I may have stimulated some of you to join me in trying to put together correctly the pieces of a small part of a very complex puzzle.

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A COMPARISON OF HISTOCHEMICAL PROPERTIES OF TUMORS WITH THE TISSUES OF THEIR ORIGIN*

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In the year 1939 Gomori published his observation on the demonstration of the enzyme alkaline phosphatase in tissue sections. This monumental achievement would have been sufficient in itself to give fame to its originator, but the continued soundness of his principles have tremendously stimulated an entirely new vista and field of endeavor. Since then many enzymes have been demonstrated histochemically so that we have today methods for alkaline and acid phosphatase, 5-nucleotidase, aldolase, adenosine triphosphate, phosphamidase, lipases, esterases, cholinesterases, sulphatase, β-glucuronidase, β-galactosidase, β-glucosidase, tyrosinase, amine oxidase, peroxidase, cytochrome oxidase, succinic and other dehydrogenases.

A great deal of work has been done on the enzymatic changes in tumors by means of homogenate techniques. Thus Greenstein (1942) found a high alkaline phosphatase activity in induced liver tumors as compared to the normal liver, and Woodard (1945) found that this also increased during the formation of this tumor. Greenstein has further elaborated on such changes found both in transplanted hepatomas and mouse tumors as to its absence, reduction and increase in a number of enzymes. Many of these enzymes have not as yet been demonstrated by histochemical means.

Fishman (1947, a, b, c) has demonstrated by homogenate techniques that the majority of human cancers had a high glucuronidase activity. The sites of these cancers were the lung, skin, breast, esophagus, stomach, colon, brain, and uterine cervix. Carcinoma of the endometrium did not show such elevations. Carruthers (1947) has also shown by homogenate methods that

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succinic dehydrogenase was higher in induced squamous cell carcinoma than in hyperplastic epidermis.

The foregoing examples indicate that differences and changes do occur in enzymes in malignant tumors. Homogenate techniques which employ grinding and extracting the tissues, centrifugation and separation of its nucleus and cytoplasmic elements such as mitochondria and microsome suffer from two distinct disadvantages. The first one is that the tumor is composed not only of malignant cells but also vessels and supporting stroma and many other unrelated cells which have an ancillary function. The second one is that by chemical procedures which may be necessary not all of the enzyme activity can be recovered. It has the advantage of giving quantitative data even though this may be obscured by admixture of other cellular elements. We believe the homogenate and histochemical methods must go hand in hand in order to utilize this knowledge to the fullest extent.

Histochemical procedures use sections and attempt to localize enzyme reactions to certain elements and cells in tissue. Demonstrable differences in enzyme activity can also be ascertained where those differences are sufficiently distinct. It does suffer from quantitation such as can be accomplished by homogenate techniques. However, recently, efforts have been made to acquire quantitative data.

Pathologists who contemplate using enzymatic histochemical reactions in their studies must orient themselves to think in terms of activity rather than stainability. Thus optimum or standard conditions must be used. The standard conditions in demonstrating activity are the incubation time, substrate concentration, and the pH. Long incubation times have a tendency to give rise to artifacts with diffusion of substrate or enzyme to other sites.

A factor that should be stressed is the loss of enzyme during preparation. This is particularly true of oxidative enzyme such as succinic dehydrogenase. In order to prevent such loss of enzyme during preparation we now freeze our tissues immediately at —70° C. in a bath of dry ice and alcohol, transfer them to a Linderstrom-Lang cryostat containing a microtome where they can be cut at —20° C. A glass window is fitted over the knife blade which permits flat sections. The method has been described by Coons, *et al.* (1951). The sections are placed on a glass slide

and kept in the cryostat until ready to go into the substrate for incubation. In this way we feel that enzymes are not denatured and will be preserved.

We have also felt for some time that this technique would enable us to quantitate enzyme activity in a single section in addition to localization. This would be highly desirable and attempt to meet the objections of both homogenate and histochemical techniques. Recently such attempts have been made by Doyle (1952), Dalgaard (1948), and Barka, *et al.* (1952), for alkaline phosphatase. The first author did an extraction procedure and the last two introduced P^{32} and Pb^{212} into the substrate which was subsequently measured by a Geiger counter. Our attempt in this direction has been made quantitating succinic dehydrogenase activity on a single section (Defendi and Pearson, 1955; Pearson and Defendi, 1954) using a new salt, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride and cutting the sections in the Linderstrom-Lang cryostat. The enzyme reaction was allowed to proceed under standard conditions, and the reduced tatrazolium was extracted from the slide by means of ethyl acetate. The amount of this reduced salt was measured in a Beckman spectrophotometer at 494 mu and compared with a known standard. The final results were equated in terms of reduced tetrazolium salt per $mm.^2/5'$/at 5 mu. We believe the extension of such techniques should be of importance as it permits visual localization and quantitation of enzyme as well as subsequent conventional staining of the same slide.

A problem which has not been fully appreciated although mentioned early by Gomori were strain and species differences in enzyme reactions in tissues of origin. That this might conceivably alter our concepts of enzyme activity in animal tumors either transplanted, experimentally produced, or spontaneous became clear in our studies on the β-glucuronidase in four transplanted mammary tumors of mice. The mammary tumors were E0771 transplanted to C57BL, H2712 to C_3H, dbrB to DBA and 1509la to A/wy. In comparison of these four tumors with each other we (Thurlow and Pearson, 1954) found that one tumor showed a high activity by homogenate techniques, one low, and the other two were intermediate. The figures expressed in terms of glucuronidase units/gm. of wet weight were high for tumor

dbrB, low for H2712 and intermediate for E0771 and 1509la. The figures were 1575 ± 90, 414 ± 27. 1384 ± 90 and 999 ± 67 respectively. However when the values for liver were expressed in their respective transplanted strains we got somewhat the same relationship. The figures for these strains were 2033 ± 82, 411 ± 27, 1625 ± 120 and 1848 ± 67. Thus in the strains with the highest glucuronidase activity the tumor activity was high and in the lowest the tumor activity was correspondingly low. Morrow, *et al.* (1949, 1950), have reported strain differences in the livers of mice. Thus one has to consider strain differences when studying tumors transplanted or arising in strains and the possible relationship between enzyme in tumor and host.

The type of histochemical reaction we used in demonstrating β-glucuronidase was one modified from Burton and Pearse (1952). The tissue enzyme splits the 8-hydroxyquinoline glucuronide leaving the hydroxyquinoline to combine with ferric sulphate to form ferric hydroxyquinoline. This is tested by potassium ferrocyanide to form a blue precipitate of ferric ferrocyanide.

A similar situation in regards to strain differences can be seen in specific and non-specific cholinesterases. In a study of 10 strains of mice (Schein and Pearson, 1955) we found that strains C57L, C_3H, RIII, C57BL, C57BR were low; 129, AKR, A were intermediate; and DBA, BALBC were high in enzyme activity. The reaction used in these studies was Gomori's simplification of Koelle's technique. Acetylthiocholine and butyralthiocholine were used as substrate for specific and non-specific cholinesterase. Preincubation with 10^{-6} M diisopropyl flurophosphate inhibited non-specific cholinesterase, whereas specific cholinesterase is inhibited only partially. On hydrolysis the liberated thiocholine reacted with the copper salt to give a relatively insoluble product, copper thiocholine, which is converted to copper sulphide by means of ammonium sulphide.

Non-specific cholinesterase revealed the high activity in the liver and particularly the pancreas where the small bile and pancreatic ducts and secretions within the ducts showed great activity in these respective organs. A number of tumors were examined, including a series of transplanted neuroblastomas, teratoma of the ovary and others with negative results.

The problem that we have been mainly interested in has been the study of enzymatic histochemical changes during carcinogenesis in induced and transplanted tumors. Wherever possible a correlation with the results of homogenate techniques was carried out. Greenstein in 1942 by the use of homogenate techniques demonstrated a high alkaline phosphatase activity in transplantable hepatoma 31. The tumor was induced in male Osborne-Mendal rats by a diet (White and Jackson, 1935) consisting of 0.06 per cent p-dimethyl aminoazobenzene (DAB) with addition of 0.5 per cent cystine and 0.5 per cent methionine. The substrate used was disodium phenyl phosphate. In 1943 Woodard studied the alkaline phosphatase activity of a series of rats fed a diet containing 20 cc. of 3 per cent DAB. The substrate used was sodium glycerophosphate. Both these investigators found a very high value in the hepatic tumor and also in livers preceding the stage of tumor formation.

We studied the histochemical localization of alkaline phosphatase during carcinogenesis in rats fed p-dimethyl aminoazobenzene (Pearson, B., *et al.*, 1950). A group of seventy-seven male Sherman rats were placed on a standard diet (Miner, *et al.*, 1943; Miller, *et al.*, 1941). Groups of animals were sacrificed after 1, 2, 3 and 4 months on the diet. The remaining animals were taken off the dye at 4 months but continued on the same diet without dye for 1 month, at which time they were sacrificed. In this way a continued spectrum of changes, both histological and histochemical, could be observed.

Our results indicated that there was a progressive increase in bile duct and biliary epithelium during this period. Newly formed vascular sprouts acompanied this process. At times this was so extensive as to form bile duct adenomas. The resulting histochemical changes revealed intense activity in the bile duct epithelium as well as the newly formed vessels. This process progressed to the fourth month when the rats were taken off the dye. Following the removal of the dye this proliferative process disappeared. During the period of the formation of cholangiofibrosis the reaction was intense around the newly formed bile ducts in the surrounding vascular endothelium and also in secretions and debris in the ducts. In the fully developed tumor the alkaline phosphatase activity was present in the vascular stroma

supporting the tumor, in secretions and debris in the lumina and wherever bile duct proliferation was still evident. The tumor cells themselves, particularly in the solid areas, showed no evidence of enzyme activity. Gomori's histochemical techniques were used with β-glycerophosphate as a substrate.

In another series consisting of 175 Sprague-Dawley rats on the same dye and diet but using Pearse's method for the demonstration of the enzyme, the results were similar. Instead of fixing material in acetone, the tissue was frozen at —70° C. and cut in the Linderstrom-Lang cryostat as previously described. The substrate used was sodium α-napthyl phosphate and the stable diazotate of 4-benzoyl amino-2; 5 dimethoxyanilene buffered at pH 9.2.

It was felt from these two series of studies that the high alkaline phosphatase previously reported by homogenate methods was due to proliferating bile ducts and vascular sprouts preceding tumor formation. In the tumor the same elements also showed high activity in addition to areas of debris and necrosis. Thus, the alkaline phosphatase plays only an ancillary role in the malignant tumor per se.

The result of these studies led us to examine further other tumore to see what role this enzyme might play. We chose a series of transplantable breast tumors, E0771, H2712, dbrB, and 1509la (Pearson, B., and Richardson, F., 1954). Previously alkaline phosphatase had been examined by Kabat and Furth (1941) in a spontaneous mammary tumor in C_3H mice which they too have no enzyme activity. However Hard, *et al.* (1948) found the enzyme in mammary tumors in C_3H, A and DBA mice. Greenstein also found high values by homogenate techniques.

In our experiments a total of 181 mice with transplanted tumors were examined. They were divided into four series, 39 E0771 tumors transplanted into C57 mice, 46 H2712 into C_3H mice, 48 dbrB tumors into DBA mice and 48 1509la tumors into A/He. A total of 1124 slides were studied using incubation times of 1 min., 5 min., 15 min., and 1 hour, with the exception of 1509la tumor which was extended to 2½ hours. The visual intensities were graded and plotted for each incubation time.

The results indicated that tumor E0771 was extremely high in enzyme activity and that the first reaction appeared in one

minute. In tumor 1509la no reaction was apparent even with incubation times of 2½ hours. The intermediary tumors were H2712 with the first reaction appearing in 5 minutes and dbrB tumor in 15 minutes. The alkaline phosphatase activity was also examined in our laboratory (Podber, E., Ryan, J. and Pearson, B., 1954) by means of homogenate technique and expressed in gamma of phosphorus per milligram of nitrogen at 30 minutes incubation time. The results as expressed were 701 ± 30 in E0771 tumor, 157 ± 3.4 in dbrB tumor, 30 ± 4.0 in H2712 tumor and only 5 ± 0.6 in 1509la tumor. This was in essential agreement with the histochemical data for high and low activity in E0771 and 1509la tumor. A discrepancy seemed apparent in H2712 tumor. When examined microscopically, it was seen that there was extremely intense activity of the luminal margin the tumor cells. It was also apparent that the lumena were not as numerous, as for example, in E0771. This suggested that the alkaline phosphatase activity might be dependent upon retention of secretory activity in the tumor.

This led us to study a series of 160 Fi hybrids between RIII females and C57L males implanted with stilbestrol pellets (Richardson, Flavia, and Pearson, B., 1954). These pellets were implanted into 8 week old animals which were sacrificed at 5, 6, 7, 8 and 9 months of age and also when palpable tumors appeared. Whole mounts were also studied for the presence of hyperplastic nodules.

The tissues were subjected to the Gomori procedure for alkaline phosphatase. Graded incubation times of 1 minute, 5 min., 15 min., and 1 hour in β-glycerophosphate were done. In this way the first reactions sites became apparent quickly and variations could be appreciated better. Animals sacrificed at 5 to 6 months showed an intense reaction in the large and medium sized ducts. The first reaction in incubation times of 1 to 5 minutes was in certain cells that correspond to the location of the myoepithelial cells. In the proliferating acini many round, varying-sized, intensely reactive granules could also be seen. Many of these were extruded into the lumen.

In mice sacrificed at seven, eight and nine months concretions of this secretory material with high enzyme activity were apparent. In areas of early tumor formation a distinct change was

apparent. The myoepithelial cells were not in evidence but there was a marked intense enzyme activity at the extreme lumenal border of the cells. Excretion of such positive material was also present in the lumen. The reaction was apparent with one minute incubation time. In the tumor where lumena were present the same phenomena were seen. Many of these tumors were complex and revealed papillary types of carcinomas. Wherever lumena were present in these radicular structures the enzyme reaction was present in the lumenal margin. Wherever the acini were large and their outer surfaces were in juxtaposition, this area also showed enzyme reaction in the vascular channels or myoepithelial cells. The vascular channels in the stalk of tumor tissue also revealed intense enzyme activity. The solid areas of tumor devoid of acini showed no enzyme activity. There seemed to be no actual variation or increase in activity during the various stages of tumor formation. There is, however, a shift in localization.

The findings suggest strongly that the role of alkaline phosphatase in the tumor has to do with endothelial or vascular surfaces, myoepithelial cells, and secretory phenomena and/or transference of substances across membranes for synthesis and secretion.

Other enzymes were examined by us during carcinogenesis and also in developed tumors. Some of these have not been studied in sufficient detail, variety or numbers to make specific conclusions. The following are some suggestions derived from a study of tumor material. Several hundred mice bearing mammary tumor E0771, H2712, dbrB and 1509la have been studied for non-specific esterase activity by Seligman's method. In all these tumors the results were negative. The liver, kidney, pancreas, bronchial epithelium and the interstitial cells of testes were rich in the enzyme. The livers of mice that had the tumors contained no less esterase than controls of the same strain without the tumor. It is possible, however, that this reaction is not sensitive enough, as subsequent studies with Pearse's modified method revealed some reaction. This method uses a-napthylphosphate and o-dianisidine as substrate buffered to pH 7.4. Although detailed studies have not been made, the reaction was tested in 125 rats fed p-dimethylaminoazobenzene and sacrificed monthly.

Wherever solid tumor appeared no reaction was present. Areas of cholangiofibrosis showed reaction, as well as some adenocarcinomatous areas. From such preliminary studies it would seem that non-specific esterase is diminished in tumors, especially those of the solid type.

We examined histochemically a number of transplanted tumors, as neuroblastoma, teratoma of ovary, and adenocarcinomas of the liver, for non-specific cholinesterase. The neural elements in the first two reacted so slightly that we felt that we could not distinguish this from non-specific background staining. Very intense enzyme reactions were present in the central and peripheral nervous system, particularly in the cerebellum. After examining a large number of these we became convinced that at least in this tumor material, possessed of primitive nervous tissue, that the enzyme reaction was negative or extremely weak. The adrenal medulla was positive. A much more striking reaction was seen in the ligamentum ovarii proprium. This was the most intense enzyme reaction that we noted even more so than the cerebellum and was not influenced by the endocrine status of the endometrium. Differences between species could not be detected. Another striking change seen was the intense non-specific cholinesterase activity in the secretory phase of the endometrium. The proliferative phase did not reveal activity. In examining a series of transplantable adenocarcinomas of the liver we noted no enzyme activity in the actively growing tumor but marked activity in areas of necrosis and necrobiosis. Both the changes in the endometrial glands and the adenocarcinoma suggests to us that during the anabolic active phase of growth both in non-malignant and malignant tissue the activity of this enzyme is low or absent and that in the catabolic phase rather marked activity was present.

In conclusion we should like to express some general ideas concerning enzymatic histochemistry in relation to tumors and the process of carcinogenesis. In order to get best results, a great deal of attention must be paid to optimal conditions under which the test is performed, such as incubation times, substrate and substrate concentration and, lastly, pH conditions. Graded short incubation times are of value in studying material in order to discriminate between highly and less highly reactive areas of a

tumor or lesion. Quantitative histochemical data should be explored more intensely as they can be correlated simultaneously with localization. The problems concerned with species and strain differences particularly in reference to β-glucuronidase and non-specific cholinesterase should be taken into consideration in dealign with tumors occurring in these animals. Our experience with alkaline phosphatase, particularly in induced liver tumors and mammary tumors, seem to indicate that the enzyme plays an ancillary role in turom formation. The role of non-specific cholinesterase during anabolic phases of growth is of interest. It is haxardous to make sweeping generalizations in regards to tumors and pathological material until a large number of cases and a variety of tumors have been carefully examined. We believe, however, that it should contribute a great deal to our understanding of tumors and tumor growth.

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AN ANTI-NEOPLASTIC FACTOR ENGENDERED BY CARCINOGENIC AGENTS AS DEMONSTRATED IN TISSUE CULTURE PREPARATIONS

MORRIS POLLARD*

When carcinogenic agents are inoculated into a susceptible host, neither the effect of the agent nor the response of the host is well understood. The latent (dormant) period represents an interval during which is evolved the cellular transformation which is referred to as a tumor. Carcinogenesis is provoked by a wide variety of physical, chemical, and biological agents, but the emerging tumor is frequently characterized by the cell type from which it stemmed.

It has been observed that tumor-resistance in ordinarily refractive animals can be dissolved by agents with anti-reticuloendothelial (RES) effect (Toolan, 1951, 1954; Foley, 1952; Hoch-Ligeti and Hsu, 1953). It has also been observed that tumors will grow in refractive animals following repeated challenge inoculations (Lewis, 1936; Lewis and Lichtenstein, 1936; Andervont, 1936). What may be interpreted as resistance, or immunity, eventually breaks down and the tumor growth proceeds uninterrupted.

Recent observations in this laboratory, using tissue culture methods, has elicited a transient anti-neoplastic (AN) response by the host to carcinogenic agents (Pollard and Bussell, 1953, 1954). Studies thus far have indicated that the AN phenomenon appears to have some chemical and genetic specificity and that the duration of the AN effect appears to coincide with the latent period of carcinogenesis. The only organ thus far studied for AN effect has been the spleen, since this organ provides easily accessible tissues with RES function.

If the AN phenomenon coincides with the latent period of carcinogenesis then this demonstrable phenomenon should be considerably shortened with transplants of tumorous tissue.

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Studies with the latter transplants have elicited a very brief and somewhat elusive AN effect in the spleens of recipient mice.

Methods. A system of dual tissue cultures was set up by which the following combinations of mouse tissue explants were studied in the same tube:

1. Carcinogenically induced sarcoma and spleen from the same animal.
2. Above tumor and spleens from genetically related and unrelated mice recently inoculated with a carcinogenic compound.
3. Above tumor and spleens from mice recently inoculated with chemically and genetically related tumor transplants.

For the induction of tumors, C3H[1] mice were inoculated subcutaneously with 0.5 mg. methylcholanthrene (MCA), 1,2,5,6, dibenzanthracene (DBA), and 3,4 benzpyrene (B.). All of these inoculums were dissolved in cooking oil. The mice were observed until tumors appeared in the injected area. Two biweekly injections with BP and DBA were required for tumor formation.

Groups of C3H mice were inoculated subcutaneously with 0.5 mg. of either MCA, DBA, or BP. At one- and two-week intervals thereafter several mice from each group were sacrificed and their spleens were individually processed for implantation in test tubes. White Swiss mice (CFW)[2] were also inoculated with the three compounds and their spleens were processed for tissue culture study.

Groups of C3H mice were inoculated subcutaneously with small particles of MCA tumor from the mice referred to above and 6 to 20 days later their spleens were processed for tissue culture study.

Tumor and spleen tissues were individually processed as follows:

Each tissue was minced with scissors so as to reduce it to pieces approximately 1 mm. in diameter. The minced tissue was washed 3 times in Hank's solution and suspended in 4 x the volume with Hank's solution and embryo extract (3=1). The processed tissues were explanted in coagulating chicken plasma onto the wall of a test tube. In the combination described above, 4 to 6 tumor particles were placed on one side of the tube and ¼ of a

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[2] Carworth Farms, New City, N. Y.

spleen mince was placed on the opposite side (Fig. I). Controls consisted of tumor explants alone. One ml. of nutrient fluid[3] was added to each tube which was then tightly stoppered. The tissues were incubated at 35° C. at a 5° angle and rotated 6 times per hour.

The explants were observed microscopically at daily intervals and changes were recorded as to growth of the explants. No tissue combination was evaluated unless the spleen tissue showed evidence of viability. Control tubes contained tumor and spleen explants alone. The nutrient fluid was adjusted to optimal pH (7.2) with $NaH\,CO_3$ when needed.

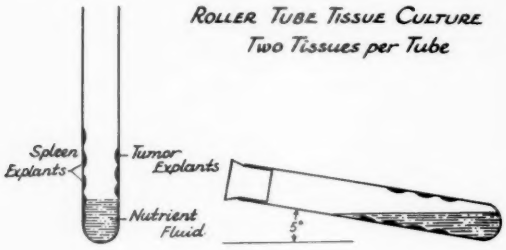

Figure I.

Results

Results previously described (Pollard and Bussell, 1953; 1954) have indicated that mice recently inoculated with one of the above carcinogenic compounds developed an anti-neoplastic (AN) factor in their spleens which was chemically and genetically related to the tumor on which they acted. Within 24 hours after explantation a corona of fibroblasts grew out from each tumor explant. When such tumor was grown in the presence of homologous spleen it grew profusely. In the presence of genetically related spleen from mice recently inoculated with a carcinogenic compound, oncolysis resulted with the spleen associated with the homologous carcinogenic agent (Fig. II). Therefore MCA tumors were not destroyed by spleens of mice recently inoculated with DBA and with BP. Genetically unrelated tissue

[3] Hank's solution, Ox. serum ultrafiltrate, chick embryo, penicillin, and streptomycin.

combinations produced no effect, regardless of the chemical relationships used (Table 1).

When the growth of spleen tissue was incompatible with tumor growth, the cytopathogenic sequence was the same in all tumors.

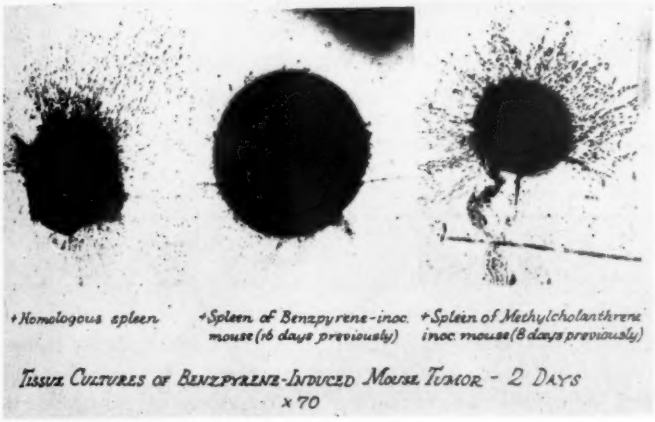
+Homologous spleen
+Spleen of Benzpyrene-inoc. mouse (16 days previously)
+Splein of Methylcholanthrene inoc. mouse (8 days previously)
Tissue Cultures of Benzpyrene-Induced Mouse Tumor - 2 Days
x 70

FIGURE II.

Usually the cells failed to grow out from the tumor explant. If some growth was observed on the first day, the tumor cells appeared pyknotic and granular by the second day.

It was very difficult to detect the AN phenomenon in spleens from mice recently inoculated with MCA tumor tissue. Tumors resulting from such inoculums appeared within the following 2 weeks, and by this time the AN phenomenon was no longer

TABLE 1

Effect of Spleen on Growth of Tumor Explants From C₃H Mice

Tumor Induced by	Growth of Tumor with Homologous Spleen	Effect of Spleens From C₃H and CFW Mice Inoculated 7–14 Days Previously with: MCA		BP		DBA	
		C₃H	CFW	C₃H	CFW	C₃H	CFW
MCA*	13/13†	1/25‡	9/9	4/4	0	2/4	0
BP	11/11	8/8	0	5/20	6/6	11/12	0
DBA	9/9	5/6	0	5/5	0	6/6	2/2

* MCA, 20-Methylcholanthrene; BP, 3,4-Benzpyrene; DBA, 1,2,5,6-Dibenzanthracene.
† Number growing/tumors tested.
‡ Growth of tumor/spleens examined; 0, not tested.

demonstrable. Some interference with tumor growth was observed with 20 of 52 spleens studied 6 to 8 days after inoculation of tumor tissue (Table 2). The AN effect was somewhat indecisive in that tumor destruction was seldom complete. The AN response failed to develop in as many mice as resulted from the carcinogenic chemical inoculations.

TABLE 2

Anti-Neoplastic Response of Mice Following Inoculation with Methylcholanthrene Tumor Mince

Days After Inoculation	5	6	7	8	20
"AN" response in spleen	4/5*	7/16	7/25	2/6	1/13

* Number of mice with AN effect/number tested.

Discussion. The AN response to carcinogenic compounds exerted a transient but marked oncoyltic effect, by which the tumor explant was thoroughly inactivated. The AN response following tumor implantation was also transient but it was shortened and weakened. In comparing the responses to the above two types of inoculums, it is evident that the tumor tissue had the capacity for accelerating the "lysis" of the AN phenomenon. If this initial host response to carcinogenesis were to be indefinitely prolonged, or revived, then a tumor may fail to appear or may undergo involution. The occasional involution of a tumor implant results in an immunity to challenge which is demonstrable by a tissue culture technique similar to that described above (Pollard and Bussell, 1953). It may be important to determine the nature of this transient host resistance, and its possible relationship to the phenomenon referred to and described as "immunological paralysis" and related phenomena (Felton and Ottinger, 1942; Felton, 1949; Morgan, *et al.*, 1953; Billingham, *et al.*, 1953; Baer, *et al.*, 1954; and Dixon and Marner, 1955).

As graphically portrayed in figure III, tumors emerge when the demonstrable host resistance to this process collapses. Under circumstances it may be naive or oversimplification to say that the tumor then assumes the role of a culture of cells growing on a nutrient medium—analogous to the growth and propagation of a bacterium on its medium. This process terminates with

exhaustion of the medium and death of the culture due to overpopulation of its invading constituents.

Considerable attention has been directed at dependent and autonomous stages in tumor development (Furth, 1954). The dependent stage of this sequence may be coincident with the antineoplastic response of the host. We still seek an explanation of the antigenic mechanism of carcinogenic agents, and most important, the nature of the cellular transformation through which a tumor cell evolves.

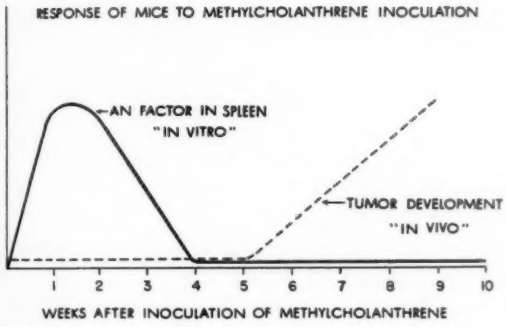

FIGURE III.

The above data offers little clarification of the carcinogenic process however, applications of the dual tissue culture technique may provide some understanding of the host response to carcinogenesis.

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CONVERSION OF FOLIC ACID TO CITROVORUM FACTOR*

B. L. Reid and J. R. Couch

Sauberlich and Baumann (1948) noted that the organism *Leuconostoc citrovorum*[1] failed to grow in a chemically defined medium containing all the known amino acids and vitamins, but the addition of small amounts of natural materials such as liver extract or yeast to the medium promoted luxuriant growth. The substance(s) in these natural materials necessary for growth of *Le. citrovorum* was termed the citrovorum factor (CF). These workers also noted that the organism responded to thymidine or to massive doses of PGA, but the response was delayed and submaximum. Sauberlich (1949) noted that rats on a PGA-deficient diet excreted little CF in the urine, but administration of PGA resulted in large increases in the CF content of the urine. Subsequently, it was noted by Sauberlich (1949*) and confirmed by Broquist, *et al.* (1950) that concentrates of CF can competitively overcome the toxicity of aminopterin (4-aminopteroyl-glutamic acid), an inhibitory analogue of PGA, for *Le. citrovorum*. It then appeared that PGA might be a biological precursor of CF and that a close chemical relationship existed between the two substances.

In a seemingly unrelated series of investigations, Bond, *et al.* (1949) provided evidence from microbiological studies for the existence of an unrecognized form of PGA in natural materials. These workers studied the toxic effect of "x-methyl PGA" for growth of *L. casei* and observed that liver extract was approximately fifteen times more active than PGA in counteracting the toxicity for growth of *L. casei* than could be accounted for on the basis of its PGA content. With the use of this inhibitor assay, highly refined concentrates of the factor from liver (designated as folinic acid) were prepared and found to be active for *Le.*

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* From the Departments of Poultry Husbandry and Biochemistry and Nutrition, Texas A. and M. College System, College Station, Texas. Presented at the Annual Symposium on Cancer Research held at the M. D. Anderson Hospital and Tumor Institute, Houston, Texas, March 15, 1955.

[1] Felton and Niven (1953) suggest that *Le. citrovorum* is a typical strain of *Pediococcus cerevisiae*.

citrovorum. It thus appeared likely that folinic acid and CF were identical.

The formula 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid has been proposed as the structure of a synthetic product (leucovorin) having CF activity (1951). Keresztesy and Silverman, (1951) and Sauberlich, (1952) have succeeded in isolating crystalline citrovorum factor from liver which has permitted comparison of the properties of the naturally occurring vitamin with leucovorin. The product obtained from horse liver by Keresztesy and Silverman was found to be approximately twice as active as synthetic leucovorin for growth of *Le. citrovorum*. Spectrographic analysis of the two products indicated that these were identical. The present belief is that the synthetic compound represents a mixture of diastereoisomers, one of which is the biologically active, naturally occurring form (Cosulich, *et al.*, 1952).

The present published evidence points to the necessity for the conversion of PGA to CF by the animal before PGA can carry out the catalytic functions and that CF either represents the active form of PGA or is more closely related to this form than to PGA. Nichol and Welch (1950) have presented evidence for the existence in liver of an enzyme(s) that is involved in the conversion of PGA to CF; of particular interest was the finding of these workers that greater amounts of CF were formed when ascorbic acid was present. Apparently ascorbic acid brought about favorable reducing conditions essential for the conversion. These workers also found that 4-amino PGA was markedly effective in blocking the conversion of PGA to CF by liver slices.

Broquist, *et al.* (1951) have reported a similar increase in CF upon incubation of liver homogenates with PGA and ascorbic acid. A report by Hill and Scott (1952) suggested that ascorbic acid increased the CF content of chick liver homogenate by activating the CF-liberating enzyme present in chick liver. These workers demonstrated the presence of bound forms of CF in yeast and liver which were liberated by conjugase enzymes from hog kidney and chick pancreas. Doctor, *et al.* (1953) reported that the conversion of added PGA to CF *in vitro* was enhanced under an atmosphere of nitrogen. These workers failed to obtain a stimulation upon the addition of ascorbic acid to the system.

The involvement of vitamin B_{12} in the formation of CF from

PGA was reported by Doctor, *et al.* (1954). These workers found that the injection of vitamin B_{12} into deficient chicks before sacrificing markedly stimulated the *in vitro* formation of CF. The addition of B_{12} *in vitro*, however, failed to produce the same results.

Nichol (1954) reported that the optimum formation of CF from PGA by cell free extracts of *S. faecalis A* required the presence of added ATP, DPN, ascorbate, Mg^{++}, glucose and a supply of formate.

Doctor, Couch, and Trunnell (1954) reported that the supernatant fraction of chick liver homogenate, after differential centrifugation, was the principal source of the PGA to CF converting enzyme system. The latter was precipitated by 40% alcohol. Homocysteine was found to consistently enhance the formation of CF. Serine was also found to stimulate the formation of CF in the presence of homocysteine and Mg^{++} by acetone powders of chick liver. Under these conditions, the glycine content was increased four-fold.

Recent work by Reid and Couch (in press) has confirmed the presence of the converting system in the supernatant portion of the homogenate and the 40 per cent ethanol precipitation of the enzyme(s) system. Ascorbic acid, however, exerted a stimulatory effect and the action of ascorbic acid was blocked through the addition of 4-amino PGA to the system. This indicates that ascorbic acid has a function in the actual formation of CF from PGA. These findings are in agreement with the earlier work of Nichol and Welch (1950) and tend to disagree with the results reported by Hill and Scott (1952) and Doctor, *et al.* (1953). The addition of ATP, DPN, ascorbate and MG^{++} stimulated the formation of CF from added PGA. The formation of CF from PGA was inhibited by the addition of glycine or cysteine to the system, while methionine, glutathione, homocystine or cystine failed to have an appreciable effect.

Reid and Couch (in press) also reported the formation of CF from PGA was stimulated by the presence of serine and homocysteine in the system. Doctor and Trunnell (1954) reported an increased excretion of CF in the urine upon the addition of cysteine and homocysteine to the diet of rats.

The results of the studies to date indicate that serine may

function in conjunction with homocysteine in supplying the formyl group for the conversion and that ATP, DPN, Mg^{++} and possibly ascorbic acid are required for the conversion to proceed at an optimal rate.

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INCORPORATION OF TYROSINE-2-C^{14} BY MALIGNANT MELANOMA

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The chemical reactions leading to the formation of melanin by the oxidation and polymerization of the amino acid tyrosine have been well established (Bu'Lock, *et al.*, Fitzpatrick, *et al.*, Raper, *et al.*, and Lerner, *et al.*). This sequence of reactions is largely controlled through the copper enzyme tyrosinase. The first two of the five oxidative steps in the formation of melanin are probably catalyzed by the same enzyme, tyrosenase. It is possible to inhibit tyrosinase activity by employing either substance that form complexes with copper or by employing analogues of tyrosine. Inhibition may also occur when substances are added that change the exidation reduction potential.

The *in vitro* uptake of radioactive tyrosine as a criterion of tyrosinase activity and melanin formation has been utilized by Fitzpatrick and colleagues (Fitzpatrick). The application of this technique as an aid in the diagnosis of human melanoma is being investigated by workers at the University of Oregon Medical School Hospital and Clinics and The University of Texas M. D. Anderson Hospital and Tumor Institute. The technique consists of incubating tissue in a phosphate buffer (pH 6.8) and tyrosine-2-C^{14}(1) solution. Subsequently, the steel planchets upon which the tissue was placed are rinsed, dried, and C^{14} actively counted in a gas flow counter.

German investigators (Poppe and Fraedrick) have reported a selective uptake of tyrosine-3-C^{14} in superficial melanotic lesions. The radioactivity at the skin surface over the lesion was measured with a thin window Geiger-Mueller counter. Almost without exception, the radioactivity was higher in the malignant melanoma than in the benign nevi. Only 5.8 per cent of 138 cases

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[1] The Tyrosine-2-C^{14} was purchased from Tracerlab, Inc.

of benign nevi showed uptake of the labeled tyrosine. An attempt was made in this laboratory to determine if experimental mouse melanoma exhibited an appreciable uptake of tyrosine-2-C^{14} similar to that reported for clinical cases.

Materials and Methods. Harding-Passey Melanoma was transferred by trochars into the groin of C3H[2] mice. After approximately three weeks when the transplants were definitely palpable, the mice were arranged in experimental groups as follows:

Experiment A. These mice were given a single dose of 0.2 microcuries of tyrosine-2-C^{14}. One animal was sacrificed in six hours following the injection, a second was sacrificed in 24 hours, a third in one week. A fourth mouse which had been given 0.5 microcuries was sacrificed after one week. The animals were sacrificed by ether inhalation and the organs were removed and sectioned (25 or 50 microns) with a freezing microtone. These sections were placed on weighed stainless steel planchets, dried *in vacuo*, and counted by a gas flow Geiger-Mueller counter. The planchets were reweighed and the counts per minute per 100 gamma dry tissue were calculated.

Experiment B. These mice were given 0.5 microcuries of tyrosine-2-C^{14} as a single injection. The urine and feces were collected daily. This material was diluted and plated on stainless steel planchets and the radioactivity was measured. Three mice were sacrificed after 24 hours. The organs were pooled and diluted 1:100 with distilled water and homogenized. A 0.2 cc aliquot of this homogenate was plated on the planchets and dried and counted as described above. These counts are given as counts per minute per 100 gamma wet weight since the dilution and concentration were known. A sample calculation is as follows:

112.2 cts per min per 0.2 cc of a 1:100 dilution of 3 melanomas $\times$ 1/20 = 5.61 cts per min per 100 gamma wet wt.

Thus 5.61 $\times$ 1820 (weight of organ in 100 gammas) = 10222 cts per min per total organs.

Some blood was also taken from the heart and placed on a weighed planchet. Following the determination of radioactivity, the planchet was reweighed and the counts per minute per 100 gamma blood was calculated.

[2] C3H mice were purchased from Jackson Memorial Laboratories, Bar Harbor, Maine.

Three mice were killed on the fifth day and were similarly treated. The next group was killed after two weeks and the organ and tumor radioactivity determined as before. The last group of three mice were sacrificed after three weeks.

Experiment C. Following these experiments a group of mice was given repeated injections of the tyrosine-2-C¹⁴. These animals were given biweekly doses of 0.2 microcuries for three and one-half weeks. A second group was given a similar biweekly dose over an eight-week interval. A control mouse which was killed three hours following an initial injection of 0.2 microcuries of labeled tyrosine was included.

One mouse in the three and one-half-week group was sacrifived three hours after the last (eighth) injection. Another was sacrificed after 24 hours; the third after 74 hours following the last injection; and the last mouse was killed after one week. The eight week animals were sacrificed 24 hours and 96 hours following the last injection.

The organs of these mice were sectioned at 25 microns and placed on weighed planchets. The calculations to counts per minute per 100 gamma were made as before.

Experiment D. In this experiment the mice were injected with 0.5 microcuries of the labeled amino acid into the tail vein. This procedure proved lethal to three of the five mice injected. One of the two remaining mice was sacrificed after 24 hours and its liver, kidney, and melanoma were homogenized and plated. The second animal was sacrificed after one week and similarly treated.

Experiment E. This experiment was similar to Experiment D except that the tyrosine-2-C¹⁴ was injected directly into the melanoma tumor. These animals were killed after 24 hours and one week respectively, and the organs processed as in Experiment D.

Experiment F. Two groups of six mice each which were given weekly doses of 0.2 microcuries of tyrosine-2-C¹⁴ were used for this experiment. One group received 0.5 mg of Melanophore Stimulating Hormone[3] in gelatin twice a week. The second control group received gelatin only. One of the mice from

[3] The Melanophore Stimulating Hormone was donated by Armour Laboratories, Chicago, Illinois.

each group was sacrificed and processed after two weeks. Two mice from each group were processed in four weeks. At six weeks the remaining mice were killed, the organs homogenized, plated and counted.

Experiment G. Radiolabeled tyrosine was given to a melanoma patient whose prognosis was poor. He was given 250 microcuries in four divided doses over four days. There was approximately a ten per cent loss because of technical difficulties

TABLE 1

Results of Experiment A
Counts/minute/100 Gamma Dry Weight of Tissue

Organs	0.2 Microcuries in ea. instance 6 Hours	0.2 Microcuries in ea. instance 24 Hours	0.2 Microcuries in ea. instance 1 Week	0.5 Microcuries in ea. instance 1 Week
Liver	38.6	2.4	2.4	22.9
Kidney	4.7	9.8	1.1	3.1
Heart	2.8	1.9	0.7	22.2
Melanoma (50 Microns)	9.3	11.3	10.9	108.9
Melanoma	4.3	31.2	2.0	133.7
Spleen	10.8	5.0	0.8	1.8
Lung	2.5	4.0	2.8	2.5
Adrenal (homog)	2.5	9.1	1.4	32.6
Stomach	11.4	2.4	0.6	63.1
Brain	...	3.9	1.6	9.4
Thyroid (homog)				15.5
total cartilage	104.6	52.2	0.5	26.3
Ovary-Uterus (homog)	...	0.5	..	...
Ovary only	...	..	23.3	...
Muscle	...	1.2	..	40.1
Skin	...	0.8	..	...
Uterus	...	..	1.2	...

* This value refers to thyroid gland only.

in withdrawing the material from a sterile vial. The urine and feces of this patient were saved and aliquots were measured for radioactivity. This patient died on the tenth day following his first injection. An autopsy was performed and aliquots of the various organs and melanoma metastases were homogenized, plated and counted.

Results and Discussion

The results of Experiment A are presented in table 1. As observed from the six-hour data, the liver contained a relatively large quantity of the radioactivity; roughly five times as much

as the melanoma. In the subsequent determinations, the liver lost most of its radioactivity. The melanoma showed the highest concentration of radioactivity; however, the kidney and adrenal were at least half as active as the melanoma at 24 hours. At the one week sacrifice, there was a considerable variation in the two samples of melanoma taken which invalidates any conclusion except that there is not a large differential of C^{14} activity in the melanoma. The results for the thyroid are not comparable in that they are from a homogenate of the thyroid cartilage with the gland rather than a microtome slice. There is a moderate amount of activity in the thyroid initiality, however.

The data for Experiment B are presented in tables 2 and 3. These results employing homogenates of the pooled organs of three mice are less subject to animal variation than the results from Experiment A. There were no differences in radioactivity between the melanoma and normal tissues. Quite often the

TABLE 2

Results of Experiment B

(Pooled organs of three mice injected with 0.5 Microcuries Tyrosine-2-C^{14})

	Counts/minute/100 Gamma Wet Weight of Tissue							
	24 Hours		5 Days		2 Weeks		3 Weeks	
Organ		Total Counts		Total Counts		Total Counts		Total Counts
Blood	2.32*	....	0.88*	....	2.17*	....	0.97*	....
Melanoma	5.61	10,222	2.07	2,640	1.5	2,284	0.645	4,700
Brain	1.00	1,256	0.39	502	1.7	2,080	0.465	574
Lung	2.10	1,115	1.01	498	2.26	1,470	0.21	126
Eyes	0.67	101.2	0.405	60.0	2.58	348	0.30	50.6
Pancreas	2.45	2,419	1.35	1,100	2.20	3,120	0.06	67.7
Uterus and Ovaries	2.47	930	0.78	395	3.14	15,300	0.31) 0.27)	125) 107)
Thyroid	1.56	158.2†	0.51	12.6	1.05	103.0†	0.465†	11.2†
Ovaries	2.68	168	1.02	73	...	....	...	....
Stomach	4.40	29.3	1.06	701	1.50	1,280	0.27	135
Kidney	6.26	8,280	2.81	3,430	2.24	3,010	0.705	881
Spleen	4.61	2,971	0.96	915	1.43	1,450	0.435	420
Heart	1.60	554.9	0.84	457	1.055	434	0.57	216
Adrenals	3.77	82.5	2.54	47.5	2.04	36.3	0.405	10.4
Liver	4.13	21,700	1.14	6,400	3.64	22,500	0.225	1,420
Intestine	4.47	44,600	1.09	11,300	2.18	25,400	2.16	25,200‡
Muscle	0.57	....	0.47	....	1.31	....	1.99	1,210
Skin	0.47	9,650	0.87	10,700	2.36	28,200	...	....
Carcass	3.65	145,000	3.38	138,000	0.09	3,784	...	....

* Values are in counts/minute/100 Gamma *Dry* Weight.
† Total Thyroid Cartilage.
‡ Ate their cage mates.

kidneys, adrenals and carcass and other tissues show a greater uptake of tyrosine-2-C^{14} than the tumor.

It may be observed from table 3 that much of the injected radioactive compound is excreted in the urine on the first day. This was believed to be the D-isomer of the dl-tyrosine-2-C^{14}

TABLE 3

Results of Experiment B
(Pooled Excretion of Radioactivity from Mice Injected with 5 Microcuries Tyrosine-2-C^{14})

Days	Mouse	Feces cts./mn./mg.	Total cts./min.	Urine cts./min./mg.	Total cts./min.
1st					
	(a)	109.6	18,200	3,283	218,000
	(b)	124.6	41,500	4,613	308,000
	(c)	100.3	33,400	2,633	175,000
	(d)	82.1	27,700	2,663	177,000
2nd					
	(d)	21.2	7,100		
3rd					
	(d)	8.2	2,740		
4th					
	(d)	8.8	2,940		
5th					
	(d)	7.6	2,540		
Total 2nd thru 5th					
	(d)	..	15,320		
2nd thru 6th					
	(b)	18.3	15,200	0.2	83.3
	(c)	19.1	15,900	5.7	237
	(d)	...	....	0.4	167
6th to 14th					
		2.7	4,500	0.2	333
14th to 21st					
		22.3	55,800*	5.7	71

* Ate their Cage Mate.

which is not utilized and is excreted. We did not actually ascertain that D-tyrosine was excreted; however, this may be contemplated for the future.

Subsequently, the radioactive material was excreted primarily in the feces at a decreasing rate. Only a minor portion was excreted in the urine following the first day. The total amount injected as calculated from plating an aliquot of the solution was 415,000 counts per minute. The amount in the mouse organs and carcass plus that lost in excretion adds up to 425,000. This is very good agreement considering the dilutions involved and the indeterminate errors of method.

The results of Experiment C in which multiple doses of tyrosine-2-C¹⁴ were used are shown in table 4. Again, there is little indication of increased concentration of labeled tyrosine-2-C¹⁴ activity in the melanoma above the mouse's organs. The table does not record the results of the eight-week group; however, they showed the same C¹⁴ distribution as the animals in the shorter three and one-half-week period.

TABLE 4

Results of Experiment C
(Repeated 0.2 Microcuries Injections)

	Counts/minute/100 Gamma Dry Weight							
Organs	3 hrs.	3½ wks. 3 hrs.	3½ wks. 24 hrs.	Total	3½ wks. 74 hrs.	Total	3½ wks. 1 wk.	Total
Blood	3.07	4.77	9.86	....	3.21	....	...	....
Blood	1.02	5.13	...	....	...	....	...	....
Brain	5.12	2.09	1.26	1260	1.68	2050	5.10	5680
Lung	1.77	3.24	1.85	931	2.38	1750	1.46	785
Eyes	...	1.09	0.84	134	0.97	171	0.75	110
Pancreas .	...	...	...	....	...	....	0.97	400
Uterus ...	1.68	5.46	4.93	286	3.92	875	3.1	1150
Thyroid ..	...	4.50	0.77	36	1.79	8	2.38	45
Ovaries ..	...	4.64	1.62	15	2.85	19	...	....
Stomach ..	2.82	7.08	4.61	3550	3.91	3270	2.30	1170
Kidney ...	3.05	4.61	9.43	10760	6.27	6210	3.32	1180
Spleen ...	2.90	4.26	3.24	2690	3.06	2470	1.23	1260
Heart	0.50	2.46	2.11	708	2.57	980	2.44	920
Adrenals .	1.26	5.65	4.73	139	3.94	79	2.36	94
Liver	1.89	5.16	4.16	23600	2.58	14000	1.58	10300
Intestine ..	1.56	7.38	...	....	...	....	...	....
Melanoma homog. ..	3.69	1.68	3.28	33500	4.50	22400	...	....
homog. ..	...	...	...	....	5.11	25500	4.64	21500
Melanoma slice	2.61	6.27	4.25	43400	4.81	24000	5.86	27200
slice	...	7.34	4.88	49800	...	....	6.31	29200
Muscle ...	0.43	0.81	1.28	1200	...	....	1.63	750

The results of Experiments D and E are shown in tables 5 and 6 respectively. There is a slight increase in the amount of radioactivity in the tumor when the C¹⁴ material is injected directly into the tumor. Even then the results are not too impressive. There was little concentration of activity in the melanoma following the intravenous injection of tyrosine-2-C¹⁴.

The fur of the animals receiving MSH was darker than the fur of the control mice as would be expected; however, as noted in

TABLE 5

Results of Experiment D
(Intravenous Injections)

Organ	24 Hours cts./min./100 Gamma*	1 Week cts./min./100 Gamma*
Blood	0.08	...
Liver	0.065	0.14
Kidney	0.26	0.54
Melanoma	0.55	0.64

* All values except Blood are counts per 100 Gamma, Wet Weight. Blood values are counts per 100 Gamma *Dry* Weight.

table 7, there was no particular change in the distribution of C^{14} between the two groups.

The patient receiving radioactive tyrosine showed multiple metastases of melanoma varying from 3 cm. in diameter to 0.5 cm. or smaller through most of his organs. A few metastases were amelanotic. The radioactivity of these metastases as well as samples of grossly normal tissue are shown in tables 8 and 9. The melanomatous portions do not show a marked increase over corresponding normal tissue although there is a slight increase in C^{14} uptake in most of the melanomatous portions.

The results of the radioactivity determinations on the urine and feces parallel the results with the mouse. Over the four days the radioactive material was being administered, there was marked radioactivity in the urine specimens. Subsequently, the radioactivity of both urine and feces was relatively small. In contrast to the mouse, more of the radioactivity was excreted in the urine than in the feces even at the later periods.

TABLE 6

Results of Experiment E
(Injection into Tumor)

Organ	24 Hours cts./min./100 Gamma*	1 Week cts./min./100 Gamma*
Blood	0.10	...
Liver	0.39	0.33
Kidney	1.00	0.58
Melanoma	2.01	1.55
Melanoma	...	2.02

* All values except Blood are counts per 100 Gamma, Wet Weight. Blood values are counts per 100 Gamma *Dry* Weight.

Since tyrosine goes to the amino acid pools where it is used in protein synthesis and is also used in the synthesis of adrenal medullary hormones as well as in the production of melanin, it is reasonable to expect that the compound would be widely distributed following administration. We plan to carry out similar experiments using dihydroxyphenylalanine (Dopa). This compound, the second intermediate in the tyrosinase system, does

TABLE 7

Results of Experiment F
(0.5 mg. Melanophore Stimulating Hormone)

	Counts/minute/100 Gamma Weight*				
Control Group Organs	2 Weeks	4 Weeks		6 Weeks	
Blood	1.15*	3.1*	4.2*	6.0	...
Liver	0.40	0.95	1.12	1.64	...
Melanoma	0.79	3.03	1.56	2.22	...
Kidney	0.91	2.22	2.66	3.20	...
MSH Group Organs					
Blood	0.82*	4.40*	...	2.7*	3.6*
Liver	0.88	1.55	1.57	1.73	1.31
Melanoma	0.89	3.50	1.44	0.40	...
Kidney	1.22	2.63	1.63	2.24	...

* All values except Blood are counts per Wet weight.
Blood values are counts per 100 gamma *Dry* weight.

TABLE 8

Results of Experiment G
(250 microcurie dose given to a patient in 4 daily doses of 62.5 microcuries)
Total counts given

Excretion Day	Urine cts./min./day	Feces cts./min./day	Total cts./min./day
1st (62.5 microcuries each day)	7.60×10^6	0.038×10^6	7.64×10^6
2nd	7.70×10^6	0.098×10^6	7.79×10^6
3rd	9.28×10^6	0.011×10^6	9.29×10^6
4th	8.41×10^6	1.516×10^6	9.93×10^6
5th	1.26×10^6	.....	1.26×10^6
6th	0.72×10^6	.....	0.72×10^6
7th	0.29×10^6	0.00	0.29×10^6
8th	0.31×10^6	0.560×10^6	0.87×10^6
9th	0.58×10^6	0.024×10^6	0.60×10^6
10th	0.30×10^6	0.014×10^6	0.31×10^6
Deceased			
Excreted Total	36.45×10^6	2.26×10^6	38.70×10^6

TABLE 9

Results of Experiment G
(Tissues at death)
(cts./min./mg.)

Organ	Normal	Melanoma	Mixed	Amelanotic
Cerebrum	2.50	6.15	...	...
Cerebrum	5.54	...	...	...
Medulla	7.29	...	...	...
Large intestine	3.92	...	...	...
Small intestine	2.09	...	...	...
Duodenum	2.90	1.75	...	...
Duodenum	6.52	4.70	...	...
Stomach	3.70	3.46	...	...
Fat	2.11	...	...	...
Pancreas	6.22	5.69	...	...
Prostate	2.34	4.30	...	...
Kidney, Right	7.40	12.50	...	...
Kidney, Left	9.45	11.60	...	...
Bladder	.60	7.20	...	...
Adrenal	7.95	9.10	...	5.30
Lymph Node	...	6.00	...	...
Lymph Node	...	8.45	...	...
Lymph Node	...	8.15	...	...
Lymph Node	...	9.6	...	...
Costal Muscle	4.75	...	...	...
Spleen	7.50	4.40	...	4.51
Lung	5.90	8.68	...	5.40
Liver	7.75	...	...	...
Liver	6.18	9.25	9.50	6.25
Heart	...	...	...	12.30
Heart	9.15	13.30	...	7.40
Testes	7.65	...	...	...
Tunica Albicans	4.2	...	...	...
Rib	2.2	...	...	...
Mediastinal Metast.	...	12.70	...	...
Thyroid	4.05	...	...	...
Blood and Tissue Fluid	2.22	...	...	...
Blood Cells	5.93	...	...	...
Gall Bladder	4.10	...	...	...
Liver, Right Lobe	7.75	10.95	...	6.66
Liver, Left Lobe	8.50	13.10	...	5.64
Skin	1.15	3.85	...	...
Vertebra	8.68	...	...	...
Sternum	...	6.74	...	...

not go to the amino acid pool and hence would be more likely to concentrate in the melanoma.

Summary. C3H mice bearing Harding-Passey melanoma were injected with single or repeated doses of tyrosine-2-C^{14} and sacrificed at varying time intervals thereafter. The various tissues as well as the malignant tumor were homogenized and the radioactivity determined.

No appreciable differences were noted in the incorporation of the radioactive labeled tyrosine by the normal or malignant tissues.

A patient with malignant melanoma was injected with multiple doses of tyrosine-2-C^{14} and, following death, samples from his organs and from the melanoma metotoses were homogenized and the radioactivity determined. Again, there was no appreciable difference in the radioactivity present in the normal or melanotic tissue.

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CALLING ATTENTION TO

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Clinical: FR Beaudette edits *Psittacosis: Diagnosis, Epidemiology & Control* (Rutgers Univ Press, New Brunswick, NJ, 55, 240pp, $4). . . . PA Cavelti analyses autoimmunologic disease (*J Allergy* 26/95/55). . . . P Fourman & GR Henry find NaCl added to diet in potassium deficiency causes edema & weight gain (*Clin Sci* 14/75/55). . . . CHW Leeksma & JA Cohen estimate average life of human blood platelets to be 8–9 days (*Nature* 175/552/Mch 26/55). . . . Medicah Research Council of Great Britain & American Heart Asso report little significant difference in treating acute rheumatic fever in children with ACTH, cortisone, or aspirin (*Brit Med J* 1/555/March 5/55). . . . JDN Nabarro discusses *Biochemical Investigations in Diagnosis & Treatment* (Little, Brown, Boston, 55, 310pp, $6). . . . R&M Silberberg find high fat diet shortens life span (*Conad J. Biochem Physiol* 33/167/55). . . . *Neuro-psychological*: RF Bales analyses interaction of people at conferences with dual leaders, "idea person" & "best-like" (*Sci Amer* 192/31/March 55).

. . . J. Brozek notes personality changes with age (*J. Gerontol* 10/194/55). . . . PBS Fowler discusses pathology of rehabilitation (*Lancet* v/DFG/*Mch*E EEQ. . . . E. Froeschels considers grammar as basic function of language-speech (*Am J Psychotherap* 9/43/55). . . . EH Gombrich gives Ernest Jones Lecture on psychoanalysis & the history of art (*Internat J Psychoanal* 35/401/54). . . . JD Holzberg & Co conclude reporting of aggression is indication of aggressive tension (*J Abn Soc Psychol* 50/12/55). . . . MA Kennard & Co attempt correlation of electroencephalography with personality structure (*EEG* N *Clin Neurophysiol* 7/29/55). . . . PF Lazarfeld & M Rosenberg well discuss *The Language of Social Research* (Free Press, Glencoe, Ill., 55, 590pp, $6.75). . . . WL Lowe makes comparative study of religious prejections A*Am J Psychotherap* 9/54/55). . . . GC Auartin reviews mental disturbances associated with ACTH & cortisone administration (*Medicine* 34/13/55). . . . EG Smith emphasizes psycholical factors in social perspectives in medicine (*Med J Austral* 1/161/Feb 5/55). . . . Sir Geoffrey Vickers discusses mental health & spiritual values (*Lancet* 1/521/Mch 12/55). . . . A Wikler shows little correlation between EEG chages & clinical effects of mescaline, morphine, & nalline (*J Nerv Ment Dis* 120/157/55). . . .

GENERAL: RJ Benford salutes *Doctors in the Sky: Story of the Aero-Medical Association* & of Louis Bauer (CC Thomas, Springfield, Ill., 55, 326 pp, $8.75). . . . FJ Brown edits *Approaching Equality of Opportunity in Higher Education* (Am Council on Education, Washington, DC, 55, 150pp, $1.5 – note series of excellent studies published by the Council). . . . P Herrmann in (*Conquest by Man* is exciting about ancient exploration (Harper, NY, 455pp, $6). . . .

JUNE, 1955

GENERAL: JE Bryan discusses *Public Relations in Medical Practice* (Williams & Wilkins Baltimore, 55, 293pp, $5). . . . TR Henry gives real information on Hiawatha & Iroquois in *Wilderness Messiah* (Sloane, NY, 55, 248pp, $4). . . . B. Kisch reminds us of forgotten medical leaders GG Valentin (1810–1883), D Gruby (1810–1898), R. Remak (1815–1865) & L Auerbach (1828–1897) (*Trans Amer Philosoph Soc* ns 44/142–317/55). . . . JH Shaw edits *Fluoridation as a Public Health Measure*Q (Amer Asso Advancement Science, Washington, 55, 232pp, $4.5). . . .

PSYCHOMEDICINE: HA Abramson edits exciting volume on chemopsychology, called *Neuropharmacology* (Josiah Macy Foundation, New York, 55, 210pp, $4.5). . . . E Brunswik & Co sympose on probabilistic theory in functional psychology (*Psychol Rev* 62/193/55). . . . O Diethelm edits *Etiology of Chronic Alcoholism* (CC Thomas Springfield, Ill, 55, 229pp, $6.5). . . . ER Goodenough directs *Toward a Mature Faith* (Prentice-Hall, NY, 55, 162pp, $3) & opines on "Our Faith & Dr Freud" (*Sat Rev*, May 14/55). . . . P Greenacre psychoanalyses Lewis Carroll (1832–1898) (*Psychoanal Quart* 24/200/55). . . . HE Himwich & Co report favorable effects from feeding sodium glutamate to psychiatric patients (*J Nerv Ment Dis* 121/40/55). . . . J Hoffman & M McDonald discuss artistic expression as part of psychotherapy (*AM J Psychotherap* 9/269/55). . . . ME Obermayer

describes *Psychocutaneous Medicine* (CC Thomas, Springfield, Ill, 55, 487pp, $8). . . . E Perry describes handling of aggressive juveniles in family group therapy (*Internat J Group Psychotherap* 5/131/55). . . . N. Reider notes demonology of current psychiatry (*Am J. Psychi.* 111/851/55). . . . JH VandenBerg discusses *The Phenomenolgical Approach to Psychiatry* (CC Thomas, Springfield, Ill, 55, 105pp, $3.5). . . . FF Yonkman edits symposium on reserpine in treating neuropsychiatric problems (*Ann NY Acad Sci* 61/1–280/55). . . .

PRECLINICAL: LG Abood suggests chlorpromazine acts by reversibly altering intramitochondrial relations regarding oxidative phosphorylation (*Proc Soc Exp Biol Med* 88/688/55). . . . K Bingold reviews significance of catalase in biological & clinical conditions (*Dtsch Med Wchnschr* 80/603/Apr 22/55). . . . A Carletti & Co find canescin from Rauwolfia conescens similar in action to reserpine (*Experientia* 11/98/55). BG Cragg offers a physical theory of axon growth (*J Cell Comp Physiol* 45/33/55). . . . R Domenjoz reviews antipyretic analgesics (*Arch Exp Path Pharmakol* 225/14/55). . . . N Jancso describes storage of proteins & vinylpolymers in histiocytes & renal epithelium (*Acta Med Acad Sci Hung* 7/173/55). . . . LM Libber describes anticoagulant action of adenosine triphosphate (*J Cell Comp Physiol* 45/103/55). . . . ER Long & Co find tubercle bacilli metabolize acetic acid & glycerol, with labelled C going to lipids, while carbon from CO_2goes to soluble proteins (*Am Rev Tuberc* 71/609/55). . . . W Schulemann reviews chemotherapy of malaria (*Arch Exp Path Pharmakol* 225/45/55(. . . . NR Shulman describes proteolytic inhibitor with anticoagulant action from human urine & plasma (*J Biol Chem* 213/655/55). . . . K Soehring & Co reviews biochemorphology of tertiary alcohols, finding bromthinyl-cyclohexanol optimum in central depression & safety (*Arzneimittelforsch* 5/161/55). . . . G Weddell & Co describe types of nerve endings in mammalian skin (*Biol Rev* 30/159/55).

CLINICAL: EA Brown reappraises reactions to mercurial diuretics (*Ann Allergy* 13/131/55). . . . R Caldeyn-Barcia & Co find morphine has no effect on uterine contractility but will relieve labor pains (*Arch Internat Pharmaco* 101/171/55). . . . CG Child reviews portal circulation, cirrhosis & portal decompression (*New Eng J Med* 252/837/May 19/55). . . . AM Cooke discusses osteoporosis (*Lancet* 1/882, 929/Apr 30, May 7/55). . . . J Hallervorden describes anatomy & pathogenesis of multiple sclerosis (*Munch Med Wchnschr* 97/509/Apr 22/55). . . . Sir Ernest Kennaway reviews problems in human cancer (*Brit Med J* 2/1107/May 7/55). . . . I Kiss & Co discuss pathology, clinical picture & epidemiology of hemorrhagic nephroso-nephritis (*Acta Med Acad Sci Hung* 7/49, 59, 83/55). . . . D Seegal edits symposium on management of patients with essential hypertension (*J Chron Dis* 1/471–588/55). . . . CJ Sullivan classifies allregic reactions A*Ann Int Med* 42/786/55). . . . J Zissler analyses action of cardiac glycosides in human hemodynamics (*Arch Kreislauf* 22/97/55).

JULY, 1955

FUNDAMENTAL: VW Adamkiewicz & Co analyse antiphlotistic action of parenteral trypsin A*Canad J Biochem Physiol* 33/332/55). . . . GA Boyd de-

scribes *Autoradiography in Biology & Medicine* (Academic Press, NY, 55, 399pp, $8.8). . . . WJ Burdette reviews significance of mutation in origin of tumors (*Cancer Res* 15/201/55). . . . E Chargaff & JN Davidson edit *The Nucleic Acids* (Aacdemic Press, NY, 55, 2 vols, 692pp, $16.8, 576pp, $14.5). . . . JA Cohen & CH Posthumus survey action of anticholinesterases (*Acta Physiol Pharmacol Neerl* 4/17/55). . . . OJ Eigsti & P Dustin Jr review *Colchicine in Agriculture, Medicine, Biology & Chemistry* (Iowa State College Press, Ames, 55, 470pp, $5). . . . H Eyring & TH Dougherty note molecular mechanisms in inflammation (*Am Sci* 43/457/55). . . . M Feughelman & Co describe molecular structure of deoxyribose nucleic acid & Nucleoprotein A*Nature* 175/834/May 14/55). . . . HR Fleck collates *Synthetic Drugs* (Cleaver-Hume Press, London 31, 55, 360pp, 70s). . . . A Fleckenstein discusses *Der Kalium-Natrium-Austausch als Energieprinzip in Muskel und Nerv* (Springer, Berlin, 55, 157pp, DM 25). . . . A Gierer analyses mechanism of alcohol dehydrogenase action (*Biochem Biophys Acta* 17/111/55). . . . P Grabar & Co discuss preparation of antileucocytic serum (*Ann Inst Pasteur* 88/548/55). . . . DM Greenberg edits *Chemical Pathways of Metabolism* (Academic Press, NY, 55, 2 vols, 460pp, $11, 383pp, $9.5). . . . C Herdan offers *Statistics of Therapeutic Trials* (Elsevier, Amsterdam, 55, 368pp, $6.). . . . MM Janot & R Goutard isolate voacangine from Voacanga africana, related to ibogaine, a methoxy indole with central effects? (*Compt rend Acad Sci* 240/1800/May 2/55). . . . G Lelkes & L Karmazzin note development of elastic fibers in tissue culture (*Acta Morph Acad Sci Hung* 5/147/55). . . . JF Manery & RA Cleghorn sympose on ion distribution in animal tissue & fluids (*Canad J Biochem Physiol* 33/453–505/55). . . . HJMuller notes genetic damage from radiation (*Science* 121/837/June 17/55). . . . C Nagato & Co show relation of frontier electron densities & carcinogenic action of aromatic hydrocarbons (*Cancer Res* 15/233/55). . . . A Nizet reviews erythropoiesis in relation to protein metabolism (*J Physiol* 47/7/55). . . . TAJ Prankerd surveys metabolism of human erythrocytes (*Brit J Haematol* 1/131/55). . . . AW Ravin discusses infection by viruses & genes (*Am Sci* 43/468/55). . . . Our RH Rigdon produces atypical cirrhosis by methylcholanthrene A*Am J. Path* 31/461/55). . . . M Saffran & AV Schally find ACTH relase by posterior pituitary extract & arterenal or by hypothalamic or cortex extract & epinephrin A*Canad J Biochem* 33/408/55). . . . MG Sevag & Co edit *Origins of Resistance to Toxic Agents* (Academic Press, NY, 55, 571pp, $12). . . . RW Smith & Co edit *The Hypophyseal Growth Hormone: Nature & Actions* (Blakiston McGraw Hill, NY, 55, 576pp, $12). . . . EA Spiegel & Co note thalamic lesions produce temporary chronotaraxis A*Science* 121/721/May 27/55). . . . WF von Oettingen surveys *The Halogenated Hydrocarbons: Toxicity & Potential Dangers* (Publ Health Service Publ 414, Washington, 55, 430pp, $2.5). . . . H Waelsch edits *Biochemistry of the Developing Nervous System* (Academic Press, NY, 55, 550pp, $11). . . . G Wald & Co fully analyse iodopsin, a carotenoid pigment in cones for dalight vision (*J Gen Physiol* 38/623/55).

CLINICAL: JC Bateman describes chemotherapy of cancer with triethylene thiophosphoramide (*New Eng J Med* 252/879/May 26/55). . . . P Endes,

J Lorine & Co note experimental & clinical malignant hypertensive syndrome (*Acta Morph Acad Sci Hung* 5/11, 111/55). . . . HC Hack discusses *The Plasma Proteins in Pregnancy* (CC Thomas, Springfield, Ill, 55, 118pp, $3). . . . JC Moir reviews history & use of ergot (*Canad Med Asso J* 72/727/55). . . . TH Newton & Co find phentolamine useful adrenolytic agent in diagnosis & management of pheochromocytoma (*New Eng J Med* 252/974/June 9/55). . . . G Nylin shows increased red cell volume in cardiac decomposition, due to increased residual blood in heart, & indicates method of measurement (*Am Heart J* 49/803/55). . . . E Podolsky edits *Management of Addiction* (Philosoph Lib, NY, 55, 413pp, $7.5). . . . P Safar is properly cautious about deliberate hypotensive anesthesia to reduce blood loss (*Surg* 37/1002/55). . . . CM Todd recommends sorbitol as arjunct to insulin in diabetic ketosis (*Austral J Exp Biol Med Sci* (32/827/54). . . . AW Woodruff gives natural history of protein deficiency anemia (*Brit Med J* 1/1297/May 28/55).

GENERAL: Note editorial on Science & Responsibility (*Nature* 175/913/May 28/55). . . . JH Hildebrand notes social responsibility of scientists (*Am Sci* 43/450/55). . . . MFA Montagu bulls eyes *The Direction of Human Development* (Harper, NY, 55, 404pp, $5). . . . Everyone sees JAMA, but may have missed BR Newell's superb *Mink Coats & Cadillacs: A Discourse on the Nourishment & Hygiene of the Soul* (158/381/June 4/55). . . . JG Sinclair probes migrant labor as a conservation problem (*Texas J Sci* 7/212/55).